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(54) Title: NOVEL RECEPTOR-TYPE PHOSPHOTYROSINE PHOSPHATASE-KAPPA

(57) Abstract

A novel receptor-type protein tyrosine phosphatase- κ (RPTP κ) protein or glycoprotein and the DNA coding therefor is expressed in a wide variety of mammalian tissues. The RPTP κ protein or glycoprotein may be produced by recombinant means. Antibodies to the protein, methods for measuring the quantity of the protein, methods for screening compounds, such as drugs, which can bind to the protein and inhibit or stimulate their enzymatic activity, are provided. Further, methods for inhibiting homophilic binding of Type II RPTP, especially RPTP κ molecules are provided.

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NOVEL RECEPTOR-TYPE PHOSPHOTYROSINE PHOSPHATASE-KAPPA

1. INTRODUCTION

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The invention in the field of biochemistry and cell and molecular biology relates to novel receptor-type protein tyrosine phosphatase protein or glycoprotein, termed RPTP κ (also known as RPTPase- κ), 10 DNA coding therefor, methods for production and identification of the protein, methods for screening compounds capable of binding to and inhibiting or stimulating PTPase enzymatic activity, methods for inhibiting homophilic binding of RPTP κ , and methods 15 for identifying compounds which are capable of inhibiting homophilic RPTP κ binding.

2. BACKGROUND OF THE INVENTION

20 Tyrosine phosphorylation of proteins is involved in an increasing number of cellular signalling events. It was originally implicated in signalling by paracrine- or autocrine-acting growth factors, and endocrine hormones such as insulin (see Yarden, Y. et 25 al., Annu. Rev. Biochem. 57:443-478 (1988) for review). It is now clear that this posttranslational modification is also involved in diverse processes such as the activation of cells of the immune system by antigens (Klausner, R.D. et al., Cell 64:875-878), 30 signalling by lymphokines (Hatakeyama, M. et al., 1991 Science 252:1523-1528 (1991); Mills, G.B. et al., J. Biol. Chem. 265:3561-3567 (1990)), and cellular differentiation and survival (Fu, X.-Y. 1992 Cell 70:323-335; Schlessinger, J. et al. 1992 Neuron 9:1- 35 20; Velazquez, L. et al., 1992 Cell 70:313-322). In view of the diversity of processes in which tyrosine

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phosphorylation is involved, it is not surprising that links are also emerging with the process of cell adhesion and cell-cell contact.

5 The identification of several growth factor receptors and retroviral oncogenes as tyrosine-specific protein kinases indicated that protein phosphorylation on tyrosine residues plays a key role in cellular growth control. This notion has recently 10 received support by the observation that the level of tyrosine phosphorylation of enzymes thought to play an important role in signal transduction (such as phospholipase C) correlates with their increased activity upon growth factor stimulation, thus 15 establishing a functional role for tyrosine phosphorylation (Ullrich, A., et al., *Cell* 61:203-212 (1990)).

Most of the processes in which tyrosine phosphorylation is implicated involve the transduction 20 of a signal through the cell membrane. In its best understood fashion, this can occur through dimerization-mediated activation of members of the receptor tyrosine kinase family by soluble ligands (reviewed in Ullrich, A. et al. 1990 *Cell* 61:203-25 212). However, modulation of receptor tyrosine kinase activity can also occur by membrane-bound ligands on neighboring cells, as in the case of the interaction 30 between the *sevenless* kinase and the *bride of sevenless* protein (Rubin, G.M. 1991, *Trends in Genetics* 7:372-376). Recently, receptor-like tyrosine 35 kinases were described with an extracellular domain similar to that of cell adhesion molecules of the CAM-family (e.g. *Axl* and *Ark* (O'Bryan, J.P. et al., 1991 *Mol. Cell. Biol.* 11:5016-5031; Rescigno, J. et al., 1991 *Oncogene* 6:1909-1913)). Such observations may 35 implicate tyrosine phosphorylation as a more broadly

used direct downstream effector mechanism for precise cell-cell recognition and signalling events. Members of the non-receptor family of tyrosine kinases have 5 also in several instances been shown to be associated with other proteins with a trans-membrane topology, examples being the association of the Lck and Fyn kinases with the CD4 protein and T-cell receptor complex components respectively (Haughn, L. et al., 10 1992 *Nature* 358:328-331; Samelson, L.E. et al., 1992 *Proc. Natl. Acad. Sci. USA* 87:4358-4362; Veillette, A. et al., 1988 *Cell* 55:301-308). However, the mechanism by which kinase activity is modulated in these instances is not understood.

15 The degree and pattern of phosphorylation of tyrosine residues on cellular proteins are regulated by the opposing activities of protein-tyrosine kinases (PTKases; ATP:protein-tyrosine O-phosphotransferase, EC 2.7.1.112) and protein-tyrosine-phosphatases 20 (PTPases; protein-tyrosine-phosphate phosphohydrolase, EC 3.1.3.48). The structural characteristics and evolution of PTKases as well as their role in the regulation of cell growth have been reviewed (Hunter, T., et al., *Annu. Rev. Biochem.* 54:897-930 (1985); 25 Ullrich, A., et al., *supra*).

2.1. PTKases

Tyrosine kinases comprise a discrete family of enzymes having common ancestry with, but major 30 differences from, serine/threonine-specific protein kinases (Hanks, S.K. et al., (1988) *Science* 241:42-52). The mechanisms leading to changes in activity of tyrosine kinases are best understood for receptor-type 35 tyrosine kinases which have a transmembrane topology (Ullrich, A. et al., *supra*). With such kinases, the binding of specific ligands to the extracellular

domain of these enzymes is thought to induce their oligomerization leading to an increase in tyrosine kinase activity and activation of the signal transduction pathways (Ullrich, A. et al., *supra*).
5 The importance of this activity is supported by the knowledge that dysregulation of kinase activity through mutation or over-expression is a mechanism for oncogenic transformation (Hunter, T. et al., *supra*;
10 Ullrich, A. et al., 1990, *supra*).

2.2. PTPases

The protein phosphatases are composed of at least
15 two separate and distinct families (Hunter, T. *Cell*,
58:1013-1016 (1989)), the protein serine/threonine phosphatases and the protein tyrosine phosphatases. This is in contrast to protein kinases, which show clear sequence similarity between serine/threonine-
20 specific and tyrosine-specific enzymes.

There appear to be two basic types of PTPase molecules. The first group is comprised of small, soluble enzymes that contain a single conserved phosphatase catalytic domain, and include (1) 25 placental PTPase 1B (Charbonneau, H. et al., *Proc. Natl. Acad. Sci.* 86:5252-5256 (1989); Chernoff, J. et al., *Proc. Natl. Acad. Sci. USA* 87:2735-2789 (1990)), (2) T-cell PTPase (Cool, D.E. et al., *Proc. Natl. Acad. Sci. USA* 86:5257- 5261 (1989)), and (3) rat
30 brain PTPase (Guan, K., et al., *Proc. Natl. Acad. Sci. USA*, 87:1501- 1505 (1990)).

The identification of a tyrosine phosphatase homology domain has raised new interest in the potential of PTPases to act as modulators of tyrosine phosphorylation (Kaplan, R. et al. 1990 *Proc. Natl. Acad. Sci. USA* 87:7000-7004; Krueger, N.X. et al.,
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1990 *EMBO J.* 9:3241-3252; see, for review, Fischer, E.H. et al., 1991 *Science* 253:401-406).

5 The second group of PTPases is made up of the more complex, receptor-linked PTPases, termed R-PTPases or RPTPs, which are of high molecular weight and contain two tandemly repeated conserved domains separated by 56-57 amino acids. RPTPs may be further subdivided into four types based on structural motifs 10 within their extracellular segments.

One example of RPTPs are the leukocyte common antigens (LCA) (Ralph, S.J., *EMBO J.*, 6:1251-1257 15 (1987); Charbonneau, H., et al., *Proc. Natl. Acad. Sci. USA*, 85:7182-7186 (1988)). LCA, also known as CD45, T200 and Ly-5 (reviewed in Thomas, M.L., *Ann. Rev. Immunol.* 7:339-369 (1989)) comprises a group of 20 membrane glycoproteins expressed exclusively in hemopoietic (except late erythroid) cells, derived from a common gene by alternative splicing events involving the amino terminus of the proteins.

Other examples of RPTPs are the LCA-related protein, LAR (Streuli, M. et al., *J. Exp. Med.*, 168:1523-1530 (1988)), and the LAR-related *Drosophila* proteins DLAR and DPTP (Streuli, M., et al., *Proc. Natl. Acad. Sci. USA*, 86:8698-8702 (1989)). Jirik et 25 al. screened a cDNA library derived from the human hepatoblastoma cell line, HepG2, with a probe encoding the two PTPase domains of LCA (*FASEB J.* 4:A2082 (1990), abstr. 2253) and discovered a cDNA clone 30 encoding a new RPTP, named He-PTP. The HePTP gene appeared to be expressed in a variety of human and murine cell lines and tissues.

A large number of members of the RPTP family, called type II RPTPs, display an extracellular domain 35 containing a combination of Ig-domains and fibronectin type III repeats (Fn-III), features typically

encountered in cell adhesion molecules (CAMS) (Gebbink, M.F.B.G. et al., 1991 FEBS Lett. 290:123-130; Streuli, M. et al., 1988 J. Exp. Med. 168: 1523-1530). An analysis of the expression pattern of several R-PTPases in the developing *Drosophila* CNS suggests some function of these molecules in aspects of axon guidance and outgrowth (Tian, S.S. et al., 1991 Cell 67:675-685; Yang, X. et al., 1991. Cell 67:661-673), an observation which might be related to the ability of R-PTPases to control the activity of src-family tyrosine kinases (Mustelin, T. et al., 1989 Proc.Natl.Acad.Sci.USA 86:6302-6306; Ostergaard, H.L. et al., 1989 Proc. Natl. Acad. Sci. USA 86:8959-8963; Zheng, X.M. et al., 1992 Nature 359:336-339). Other studies have raised the possibility that certain R-PTPases may function as tumor suppressor genes, e.g. by controlling contact inhibition (LaForgia, S. et al., 1991 Proc. Natl. Acad. Sci. USA 88:5036-5040). Elevation of cellular phosphotyrosine may occur through mechanisms other than the activation of a tyrosine kinase itself. For instance, expression of the *v-crk* oncogene, though not a tyrosine kinase, induces the phosphorylation of tyrosine residues through a poorly understood mechanism (Mayer, B.J. et al. (1988) Nature 332, 272-275). Potentially, such an outcome could result from either mutation of the substrate or through a general decrease in cellular phosphatase activity, especially in view of the normally high turnover rate of cellular tyrosine-phosphate (Sefton, B.M. et al. (1980) Cell 20:807-816). The latter possibility is suggested by the demonstration that tyrosine phosphatase inhibitors can "reversibly transform" cells (Klarlund, J.K. Cell 41: 707-717 (1985)). PTPases could therefore act as recessive oncogenes.

While we are beginning to understand more about the structure and diversity of the PTPases, much remains to be learned about their cellular functions.

5 Thus, a better understanding of, and an ability to control, phosphotyrosine metabolism, requires knowledge not only the role of PTKase activity, but the action of PTPase enzymes as well. It is clear in the art that further delineation of structure-function 10 relationships among these PTPases and RPTP membrane receptors are needed to gain important understanding of the mechanisms of cell growth, differentiation, and oncogenesis.

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3. SUMMARY OF THE INVENTION

The present inventors have conceived of a role for RPTPs in cellular control mechanisms, both as 20 potential anti-oncogenes, and as effectors in a newly discovered mechanism of transmembrane signalling. They therefore undertook a search for individual RPTP genes and proteins in mammals, including humans, which are potentially involved in such processes, and 25 describe herein the identification of a novel, widely expressed member of the RPTP family, RPTP κ , in both mice and in humans which has a transmembrane topology. The novel human RPTP κ disclosed herein consists of two 30 associated subunits whose expression is modulated by cell-to-cell contact, and, in a manner analogous to receptor tyrosine kinases, is subject to direct regulation by extracellular ligands which bind to the extracellular portion. Further, as is demonstrated in the Working Example presented in Section 15, infra, 35 RPTP κ is shown to homophilically bind other RPTP κ molecules.

The present invention thus provides a mammalian, preferably a human, receptor-type protein tyrosine phosphatase- κ (RPTP κ) protein or glycoprotein molecule, a functional derivative of the RPTP κ , or a homolog of the RPTP κ in another mammalian species. When the RPTP κ molecule is of natural origin, it is substantially free of other proteins or glycoproteins with which it is natively associated. RPTP κ is naturally expressed in mammalian brain and is developmentally and anatomically regulated. It is also expressed in other mammalian tissues. The RPTP κ molecule of the present invention may also be prepared by chemical synthesis or by recombinant means. Thus, the substantially pure RPTP κ protein or glycoprotein of the present invention may be produced by biochemical purification of the protein or glycoprotein of natural origin or by production using chemical synthesis or by recombinant expression in prokaryotic or eukaryotic hosts.

In particular, the invention is directed to a mammalian RPTP κ protein or glycoprotein having the amino acid sequence of RPTP κ shown in FIG. 3 (SEQ ID NO:1). In another embodiment is provided a functional derivative thereof. Preferably, the RPTP κ is of human origin, and has the amino acid sequence SEQ ID NO:2, as shown in FIG. 15(1)-(3).

The invention is further directed to a nucleic acid molecule, preferably DNA, which may consist essentially of a nucleotide sequence encoding a mammalian RPTP κ having the nucleotide sequence SEQ ID NO:3 (FIG. 1(1) - 1(5)). Preferably, the nucleic acid molecule consists essentially of a nucleotide sequence encoding human RPTP κ and having the nucleotide sequence SEQ ID NO:4 or encodes a functional derivative thereof. The DNA molecule is

preferably cDNA or genomic DNA. The invention is further directed to the DNA molecule in the form of an expression vehicle, as well as prokaryotic and 5 eukaryotic hosts transformed or transfected with the DNA molecule.

Also included in the present invention is a process for preparing a RPTP κ protein or glycoprotein, or a functional derivative thereof, comprising:

- 10 (a) culturing a host capable of expressing the protein, glycoprotein or functional derivative under culturing conditions,
- (b) expressing the protein, glycoprotein or functional derivative; and
- 15 (c) recovering the protein, glycoprotein or functional derivative from the culture.

This invention is also directed to an antibody, either polyclonal, monoclonal, or chimeric, which is specific for the RPTP κ protein or glycoprotein.

20 This invention is also directed to a method for detecting the presence of nucleic acid encoding a normal or mutant RPTP κ in a cell or in a subject, comprising:

- 25 (a) contacting a cell or an extract thereof from the subject with an oligonucleotide probe encoding at least a portion of a normal or mutant RPTP κ under hybridizing conditions; and
- (b) measuring the hybridization of the probe to the nucleic acid of the cell, thereby detecting the presence of the nucleic acid, preferably DNA.

30 The DNA can be selectively amplified, using the polymerase chain reaction, prior to assay.

The invention is further directed to a method for detecting the presence, or measuring the quantity of 35 RPTP κ in a cell or cells, comprising:

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5 (a) contacting said cell or an extract thereof with an antibody specific for an epitope of RPTP κ ; and (b) detecting the binding of the antibody to the cell or extract thereof, or measuring the quantity of antibody bound,

thereby detecting the presence or measuring the quantity of the RPTP κ .

10 The present invention is also directed to methods for identifying and isolating a compound capable of binding to RPTP κ from a chemical or biological preparation comprising:

15 (a) attaching RPTP κ , or the ligand-binding portion thereof, to a solid phase matrix; (b) contacting the chemical or biological preparation with the solid phase matrix allowing the compound to bind, and washing away any unbound material; (c) detecting the presence of the compound bound to the solid phase matrix; and, for purposes of 20 isolation, (d) eluting the bound compound, thereby isolating the compound.

25 Further, the present invention includes a method for identifying an agent capable of stimulating or inhibiting the phosphatase enzymatic activity of RPTP κ , comprising:

30 (a) contacting the agent with the RPTP κ in pure form, in a membrane preparation, or in a whole live or fixed cell; (b) incubating the mixture in step (a) for a sufficient interval; (c) measuring the enzymatic activity of the RPTP κ ; (d) comparing the enzymatic activity to that of the RPTP κ incubated without the agent, 35 thereby determining whether the agent stimulates or inhibits the enzymatic activity.

Still further, the invention provides methods for inhibiting the homophilic binding of Type II RPTP, 5 preferably the homophilic binding of RPTP κ , provides methods for identifying agents capable of inhibiting such Type II RPTP homophilic binding, and methods for inhibiting endogenous Type II RPTP homophilic binding in mammalian subjects.

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4. DESCRIPTION OF THE FIGURES

FIG. 1(1) - 1(5) shows the complete nucleotide sequence and amino acid sequence of murine RPTP κ . The 15 signal peptide, A5 homology region, transmembrane domain, and PTPase domains are designated by brackets.

FIG. 2 is a schematic representation of the various RPTP κ cDNA clones isolated, and the proposed domain structure of the RPTP κ protein. Translational 20 start and stop codons as well as restriction sites mentioned in the text are indicated. The vertical arrow indicates the position of the furin cleavage site. TM: transmembrane segment.

FIG. 3 shows the predicted amino acid sequence of 25 the RPTP κ precursor protein. The putative signal peptide and transmembrane (TM) segment are underlined. The two tandem phosphatase domains are boxed (PTP-1, PTP-2). The proteolytic cleavage site (RTKR 640-643) is printed in bold, and the Ig-like domain (Ig, 30 214-270) shown in bold italic characters. A5: homology to A5 surface protein (Takagi, S. et al., 1991 *Neuron* 7:295-307); FN-III: fibronectin type III repeats. The Genbank accession number for the cDNA sequence is L10106.

35 FIG. 4 shows a proposed alignment of the four FN-III repeats of RPTP κ and domain 7 of human fibronectin

(Kornblhtt, A.R. et al., 1985 EMBO J. 4:1755-1759). Residues most typically conserved in FN-III repeats are highlighted in bold. Residues identical in three or more out of the five aligned sequences are indicated with an asterisk. This region of the protein also contains clearly detectable homology to LAR, *Drosophila* PTPase 10D, and *Drosophila* neuroglian, all of which have been reported to contain FN-III repeats.

10 FIG. 5 shows an alignment of the N-terminal domains of RPTP κ and mRPTP μ with the cell surface protein A5 (Takagi et al., *supra*). Numbers indicate the first residue of the respective proteins shown in the alignment. Residues marked as consensus are identical between A5 and RPTP κ , or between A5 and mRPTP μ . Conservative substitutions are present but not shown. Residues in bold (C,W) define a possible Ig-like domain structure.

15 20 FIG. 6 shows the expression of RPTP κ mRNA in adult tissues using Northern blot analysis of poly(A)+RNA from various mouse tissues. The entire cDNA fragment from clone λ -604 was used as a probe. A similar pattern of hybridization was seen using as a probe the λ -50 cDNA clone and the N-terminal half of 25 the λ -35 cDNA clone. Positions of RNA molecular weight markers, in kb, are indicated on the left side.

20 25 FIG. 7 is a gel pattern showing the immunoprecipitation of the RPTP κ protein. HeLa cells transiently transfected by the calcium phosphate technique with an RPTP-ic expression vector (+) or an empty expression vector (-) were analyzed by radio-30 immunoprecipitation using antiserum 116 directed 35 against a synthetic peptide corresponding to residues 60 to 76 in the extracellular domain. The

immunoprecipitation was performed in the absence (-) or presence (+) of 20 μ g of the immunogenic peptide (a- κ : anti RPTP κ antiserum 116; pre: corresponding preimmune serum). Positions of protein molecular weight standards (expressed in kDa) are indicated on the left side of the autoradiogram.

FIG. 8 shows the protein tyrosine phosphatase activity of anti-RPTP κ immunoprecipitates. The RPTP κ protein was immunoprecipitated from transiently transfected COS cells using anti-N-terminal antibody 116 or corresponding preimmune serum. The PTPase activity in the immune complexes was analyzed in the absence (-) or presence (+) of vanadate. The amount of radioactivity released as inorganic phosphate is expressed as the percentage of the total input radioactivity. A representative of several experiments is shown.

FIG. 9 shows RPTP κ immunoreactive species in COS cells, and effect of Endo F treatment on SDS-PAGE mobility. Total lysates from mock or RPTP κ transfected COS cells were treated or not with Endo F. The lysates were resolved by SDS-PAGE and immunoblotted with anti-N-terminal antibody 116 (left panel) or anti-cytoplasmic antibody 122 (right panel). The 95 kDa band in panel B also seen in mock-transfected cells is presumably due to fortuitous reactivity of antiserum 122 and not relevant to the analysis. No such protein species was detectable using an antiserum raised against the same antigen in a different rabbit. FIG. 10 shows results of a pulse-chase analysis of RPTP κ processing. Mock-transfected cells (lanes 1 and 2) and cells transfected with a wild type RPTP κ expression vector (lanes 3 to 6) were metabolically labeled with [35 S]-methionine (200 [μ Ci/ml) for 15 minutes ("pulse") and

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chased for the time-periods indicated.

Immunoprecipitation was performed using antiserum 116.

Arrows indicate the positions of the 210 kDa RPTP κ precursor and the 110 kDa N-terminal cleavage product.

5 FIG. 11 shows the effect of mutagenesis of the furin cleavage motif RTKR on RPTP κ processing. Total lysates from mock-transfected COS cells, cells expressing wt RPTP κ , or RPTP(κ) carrying a mutation in 10 the furin cleavage motif RTKR (CM κ) were resolved by SDS-PAGE. Immunoblotting was performed using anti-N-terminal antiserum 116 (left panel), or anti-cytoplasmic antiserum 122 (right panel).

15 FIG. 12 shows the co-immunoprecipitation of the RPTP κ processing products. Total lysate from mock or wild type RPTP κ transfected COS cells was subjected to immunoprecipitation using anti-N-terminal antiserum 116, and the precipitate immunoblotted with anti-cytoplasmic antiserum 122. As a control, total lysate 20 from RPTP κ transfected cells was loaded in the right lane on the immunoblot.

25 FIG. 13 is a series of micrographs showing the *in situ* hybridization analysis of RPTP κ expression during development and in the adult CNS.

30 Left panel shows localization of RPTP κ mRNA in the rat at embryonic day 18. CTX, cerebral cortex; MB, midbrain; SC, spinal cord; L, liver; K, kidney; I, intestine. Right panel shows localization of RPTP κ mRNA in a sagittal section of rat brain at postnatal day 6. CTX, cerebral cortex; CB, cerebellum; DG, dentate gyrus. In the cerebral cortex, particularly in the occipital region, the labeling is not uniform in all the cortical cell layers. In the hippocampal formation labeling is more intense in the dentate gyrus and in CA3. In the cerebellum, the most intense labeling is seen in the external granular cell layer.

FIG. 14 is a molecular model describing the processing of the R-PTP- κ precursor protein. A furin-like endoprotease cleaves the 210 kDa precursor protein, after which both cleavage products (110 and 100 kDa) remain associated. No suggestions as to the mechanism of association are intended. The numerals 116 and 122 designate the sites of epitopes recognized by antisera described in the text.

FIG. 15(1)-15(3) shows the nucleotide sequence of the human RPTPK κ (SEQ ID NO:4), designated MCP7, and its derived amino acid sequence (SEQ ID NO:2).

FIG. 16. A comparison of the amino acid sequence of RPTPK κ to the amino acid sequence of hRPTP μ . Lack of designation of an amino acid in hRPTP μ indicates identity to the MCP7 sequence. The putative signal peptide, the cleavage site and the transmembrane region are underlined, the beginning of each FN-III repeat is indicated. Both PTPase domains are shown with a shaded background.

FIG. 17 shows a Northern blot analysis of MCP7 mRNA from human tissues. Poly(A)+RNA (4 μ g per lane) prepared from the indicated tissues was probed with a 32 P-labeled fragment corresponding to the extracellular domain of MCP7. The blots were applied for a 5 day exposure using an intensifying screen.

FIG. 18 shows a Northern blot analysis of MCP7 mRNA from several different human breast cancer cell lines. Poly(A)+RNA (4 μ g per lane) prepared from the indicated cell line was probed as in FIG. 15 and the blots similarly exposed.

FIG. 19 shows gel patterns indicating the transient expression of MCP7 mRNA in transfected cells. Cells of the 293 line were transfected with a MCP7 expression vector (or an empty vector as a control), metabolically labeled for 24 hours with [35 S]

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methionine and incubated with an anti-N-terminal antiserum 116. Cells were washed, lysed and protein-antibody complexes were removed by protein-A 5 sepharose. Left panel shows a SDS-PAGE gel of immunoprecipitates. Right panel shows Western blots of SDS-PAGE gels of lysates of cells transfected by MCP7-CMV (lane 1) or "empty" CMV (lane 2) and immunoblotted with the anti-N-terminal antiserum 116.

10 FIG. 20 shows Western blot patterns indicating co-expression of MCP7 with different RTKs. Semiconfluent 293 cells were transfected with expression plasmids encoding the indicated RTK together with either an equal amount of MCP7 15 expression vector or a control plasmid. After stimulation with the appropriate ligand: stem cell factor (SCF) for the p145^{rtk} RTK; epidermal growth factor for all other RTKs; insulin for I-R, cells were lysed, aliquots run on SDS-PAGE and transferred to nitrocellulose. Proteins were immunoblotted with 20 anti-phosphotyrosine antibody 5E.2. Molecular mass markers are indicated.

25 FIG. 21 shows Northern blots indicating the relationship between MCP7 mRNA levels and the state of cell confluence in SK-BR-3 cells (left panel) and HT-29 cells (right panel) in culture. Poly(A)+ RNA (4 μ g per lane) was prepared from cells obtained at different levels of confluence (lanes 1 and 4: 40%; lanes 2 and 5: 70%, lane 3 and 6: 100%) and was probed 30 with a ³²P-labeled DNA probe corresponding to the extracellular domain of MCP7 (upper blots) and with a fragment coding for GAPDH (lower blots).

35 FIG. 22A. Expression of the R-PTPK protein in transfected S2 cells. Detergent lysates were prepared from transfected cells, resolved by SDS-PAGE, and immunoblotted with an antiserum directed against the

extracellular domain of the R-PTP κ protein (Y.-P. Jiang et al. Mol. Cell. Biol. 13, 2942 (1993)). Lanes: 1, R-PTP κ anti-sense transfected cells, not 5 heat-shocked; 2, anti-sense transfected after heat-shock; 3, sense transfected cells, not heat-shocked; 4, sense-transfected cells after heat-shock; 5, lysate from COS cells transiently transfected with an R-PTP κ expression vector (Y.-P. Jiang et al. Mol. Cell. Biol. 10 13, 2942 (1993)). Molecular weight standards are indicated in kilodaltons.

The entire RPTP κ cDNA was introduced in both orientations as a HpaI/EcoRV fragment into the HpaI site of a derivative of the pCasper expression vector 15 containing the hsp70 promoter, and the resulting construct co-transfected with the pPC4 plasmid (conferring α -amanitin resistance) into S2 cells using calcium phosphate precipitation. Pools of stably transfected cells were selected in the presence of 20 5 μ g/ml α -amanitin for three weeks. Transfected cells were heat-shocked at 37°C for 30 minutes and allowed to recover for 2 hours. Adherent cells were collected, and washed twice in BSS (Kramer, H. et al., 1991, Nature 352:207; Snow, P. et al., 1989, Cell 59:313).

25 FIG. 22B. Photographs of transfected cell populations after heat-shock induction and aggregation for 2 hours. Left panel, control (anti-sense transfected) cells; right panel, cells transfected with an expression vector carrying the R-PTP κ cDNA in 30 the sense orientation; insert: higher magnification of a typical aggregate.

FIG. 22C. Time-course and quantitation of aggregation by Coulter-counting of above-threshold particles. Open squares: anti-sense vector 35 transfected cells, uninduced; full squares, idem, induced; open circles, cells transfected with an

expression vector containing the R-PTP κ cDNA in the sense orientation, uninduced; full circles, sense, induced. Standard errors are indicated by error bars.

5 Adherent, transfected cells were collected, washed twice with BSS, resuspended in BSS at a concentration of 4×10^6 cells/ml, and incubated in Coulter-Counter vials on a rotary shaker for 2 hours at 100 rpm at room temperature. For each time-point, 10 1 ml was counted using the Coulter-counter with the following settings: 1/amplification=4; threshold=10; 1/aperture current=32.

FIG. 22D. Effect of deletion of the intracellular domain of the R-PTP κ protein, and 15 mutation of the furin cleavage site. Parental S2 cells were transiently transfected with expression vectors encoding an R-PTP κ cDNA in which the furin cleavage site had been mutated (CM) (Y.-P. Jiang et al. Mol. Cell. Biol. 13, 2942 (1993)), a cDNA encoding 20 a catalytically inactive deletion mutant of R-PTP κ lacking most of the intracellular (PTPase) domain (Δ -PTP), or a wt R-PTP κ cDNA (wt). For the deletion mutant, a cDNA encoding a truncated, catalytically 25 inactive form ($\Delta\kappa$) of RPTP κ was constructed by restriction digestion with BspEI and Klenow fill-in of the wild type cDNA. This leads to the introduction of 30 a stop codon after amino acid residue 1083, and the generation of a protein lacking the cysteine residues essential for catalysis in the two intracellular catalytic homology domains of RPTP κ . Cells were heat-induced 72 hours after transfection, subjected to aggregating conditions for 2 hours, and above-threshold aggregates counted with a Coulter-counter. 35 Error bars indicate standard errors. Transfected, but non heat-shock induced cells behaved as untransfected parental cells. The apparent differences in

aggregation intensity between the different forms of R-PTP κ may reflect protein expression levels. The numbers provided by Coulter-counter counting actually provide an underestimation of the amount of aggregation as determined by visual inspection and counting of aggregates, since only large particles above a certain threshold size are scored by the Coulter-Counter.

10 FIG. 23. Aggregates consist solely of cells expressing the R-PTP κ protein. Two different cell populations, one of which had been labeled with the fluorescent dye dil (J. Schlessinger et al. *Science* 195, 307 (1977), were allowed to co-aggregate and the 15 resulting aggregates inspected by visible and fluorescence microscopy. dil-fluorescence is white in the photographs.

Left: a pool of R-PTP κ expressing cells was allowed to aggregate in the presence of an equal number of dil-stained R-PTP κ -negative cells.

Middle: R-PTP κ expressing cells were stained with dil and allowed to aggregate in the presence of unstained R-PTP κ -negative cells.

Right: mixture of stained and unstained R-PTP κ -positive cells.

25 In each case, ten aggregates were randomly localized under visible light only. Subsequent inspection under U.V. light consistently showed the staining pattern exemplified in the photographs. dil dye (Molecular 30 Probes, Inc.) was added to the growth medium at a concentration of $3.2\mu\text{M}$ during heat shock, and washed away prior to recovery and assay. 2×10^6 cells of each population were mixed and allowed to co-aggregate in a total volume of 1 ml.

35 FIG. 24. Adhesion of R-PTP κ transfected cells to a surface coated with recombinant purified R-PTP κ

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extracellular domain protein. R-PTP κ -negative, 1, or positive, 2, S2 cells, or R-PTP κ -negative, 3, and positive, 4, L6 cells were incubated with a surface 5 partially coated with the K2AP protein (circle), and the adherent cells fixed and stained. Amino acids 1- 639 of the RPTP κ proprotein were fused in-frame with 10 human placental alkaline phosphatase in the vector pBacblue III (Invitrogen) by a series of appropriate cloning steps. Recombinant virus was generated and used to infect High-Five cells for production of the 15 K2AP fusion protein using standard procedures. A secreted alkaline phosphatase (AP) control protein was generated in L6 myoblast cells by stable transfection with a modified version of the AP-TAG vector encoding 20 a fusion protein of AP with a signal peptide. Both proteins were affinity purified by elution from an anti-alkaline phosphatase monoclonal antibody (Medix Biotech) column using 100 mM diethanolamine pH 11.5, or 50% ethylene glycol, dialyzed against PBS, and 25 stored at 4°C. The K2AP and AP proteins were approximately 90% and 50% pure, resp. as determined by silver staining. To generate a mammalian cell line expressing the RPTP κ protein, an MJ 30-based RPTP κ expression vector was co-transfected with pSVneo into 30 L6 cells, and individual clones surviving G418 selection screened for expression using immunoblotting. This procedure did not detect endogenous RPTP κ protein in the parental L6 cells. The expressed protein underwent appropriate furin 35 cleavage as described (Jiang, Y.-P. et al., 1993, Mol. Cell. Biol. 13:2942). For adhesion assays, 4 μ l aliquots of protein samples (20 μ g/ml) were spotted on 35 mm 35 bacteriological Petri dishes and incubated at room temperature for 30 minutes. The solutions were

removed by aspiration, and the surface of the entire plate blocked with 1% heat-inactivated BSA for 60-90 minutes. The plates were incubated with a suspension 5 of S2 cells (4×10^6 /ml) in BSS with shaking (50 rpm) for one hour at room temperature, or with L6 cells in S-MEM (2×10^6 /ml) without shaking, at 37°C, washed three times with PBS, fixed and stained.

10

5. DETAILED DESCRIPTION OF THE INVENTION

Through the use of recombinant DNA methods, the present inventors have identified novel mammalian 15 receptor-type (transmembrane) protein tyrosine phosphatases (PTPase; EC 3.1.3.48). In view of its receptor-like structure, and the likelihood that it is part of a family, the inventors have termed this protein, RPTP κ (receptor protein tyrosine phosphatase-
20 κ). The family is designated herein as the "RPTPs." Human RPTP κ has 1444 amino acids (SEQ. ID NO:2).

Human RPTP κ (also designated MCP7) has an extracellular domain composed of one "MAM" domain, which is a sequence motif spanning about 170 amino 25 acid residues, which was recently established by comparison of several functionally diverse receptors (including RPTP μ and the A5 protein) and is thought to play a role in cell adhesion (Beckmann & Bork, 1993, TIBS 18:40-41). The extracellular domain further 30 includes one Ig-like, and four FN-type III-like segments. It therefore shares structural features with some cell adhesion molecules, permitting the classification of RPTP κ into the type II PTPase class.

The cDNA cloning of human RPTP κ and the complete 35 DNA and amino acid sequences of human RPTP κ and its murine homologue are described herein. Northern

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analysis has been used to identify the natural expression of the protein in various cells and tissues. A partial cDNA clone of the catalytic domain of RPTPk/HPTPk has been previously described (commonly 5 assigned U.S. Patent Application Serial No. 07/654,188, from which the present application claims priority; Kaplan et al., *Proc. Natl. Acad. Sci.* 87:7000-7004 (1990); Krueger et al., *EMBO J.* 9:3241-10 3252 (1990)).

10 RPTPk has been shown to be expressed in anatomically distinct regions of rat brain and its expression has been found to be developmentally regulated.

15 Remarkably, in addition to being composed of intracellular domains having enzymatic activity, the receptor family to which RPTPs belong includes transmembrane proteins having and N-terminal extracellular domains, analogous to the tyrosine kinase enzyme family (Tonks, N.K. et al. (1988) 20 *Biochemistry* 27:8695-8701; Charbonneau, H. et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:7182-7186; Streuli, M. et al. (1988) *J. Exp. Med.* 168:1523- 2530; Streuli, M. et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:8698-8702). The present inventors have therefore 25 concluded that ligands in the extracellular environment can control the activity of this membrane-associated subclass of PTPases.

30 Further, results presented in the current invention demonstrate that Type II RPTPs undergo homophilic binding, i.e., Type II RPTP receptor molecules have the ability to bind to each other. Homophilic binding, as defined here, may include 35 intercellular binding and/or binding of at least two Type II RPTP receptor proteins present on the surface of the same cell. In addition, homophilic binding may

include not only binding of identical Type II RPTP molecules to each other, for example binding of at least two RPTP κ molecules to each other, but may also 5 include the binding of any two Type II RPTP molecules to each other, such as, for example, the binding of RPTP κ to another Type II RPTP molecule. As demonstrated in the Working Example presented in Section 15, below, RPTP κ undergoes intercellular 10 homophilic binding to other RPTP κ molecules. This result represents the first example of such a homophilic binding mechanism observed within the RPTP family of molecules, and provides a link between cell-cell contact and cellular signaling events involving 15 tyrosine phosphorylation.

RPTP κ is useful in methods for screening drugs and other agents which are capable of activating or inhibiting the PTPase enzymatic activity, and thereby affecting major pathways of cellular 20 metabolism. By attaching an intact RPTP κ , or the ligand-binding portion thereof, to a solid phase matrix, an affinity probe is created which can be used to screen biological products or chemical agents for their capacity to interact with the receptor on the 25 basis of their binding activity. Bound material can then be eluted from the affinity probe in purified form.

RPTP κ is also useful in methods for screening drugs and other agents which are capable in inhibiting 30 Type II RPTP homophilic binding, and thus affecting major processes involving, but not limited to, cell-cell and/or cell-ECM (extracellular matrix) interactions. By attaching an intact Type II RPTP such as RPTP κ , or an extracellular domain thereof, to 35 a solid matrix, drugs or other agents may be screened for their ability to bind the RPTP. Those agents

which bind the RPTP with specificity may be eluted off the solid phase matrix in purified form and further tested for their ability to inhibit RPTP homophilic binding. Note that it is intended to be within the scope of this invention that the inhibition of RPTP homophilic binding described herein refers to not only the binding of at least two identical Type II RPTP molecules, such as at least two RPTP κ molecules to each other, but also to binding of any Type II RPTP class of molecules to each other, such as, for example, the binding of RPTP κ to another Type II RPTP molecule. Potential agents which may inhibit such Type II RPTP binding may include, but are not limited to, soluble portions of Type II RPTP extracellular domains, antibodies directed against Type II RPTP extracellular domain epitopes, or small synthetic molecules. RPTP extracellular domains may include all or any inhibitory portion of the MAM, Ig, and/or fibronectin Type III (FN-III) domains, as well as peptides which include the HAV, and/or the RXR/LR consensus sequences, as described below. Any of the inhibitory compounds which inhibit homophilic RPTP binding may but are not required to modulate the phosphatase activity of the RPTP molecules whose binding capability is affected.

Further, the ability of a compound to inhibit Type II RPTP κ homophilic binding may be tested in a variety of ways. RPTP κ will be used as an example, but it should be kept clear that such techniques may be used for any Type II RPTP molecule. RPTP κ , or an extracellular domain thereof, may first be immobilized by attachment to a solid matrix, using techniques well known to those of ordinary skill in the art. Such a solid matrix may include but is not limited to a petri dish, microtiter well, or a glass, plastic or agarose

bead. Second, RPTP κ , either in a purified protein form or, alternatively, present in a cell membrane preparation or present on the surface of an intact cell, may be incubated in the presence of the solid matrix together with a compound of interest. The ability of the compound to inhibit RPTP κ homophilic binding to the solid matrix may then be assayed by determining if RPTP κ molecules bind the immobilized molecules. Such a determination may be accomplished using a variety of techniques well known to those of ordinary skill in the art and include, but are not limited to the labelling of the RPTP κ present in purified form, in a cell membrane preparation, or in an intact cell. Alternatively, a compound of interest may be tested by incubating RPTP κ -expressing cells in the presence of the compound of interest and subsequently assaying the ability of the cells to undergo aggregation. Aggregation assays may include, but are not limited to directly counting aggregates using the aid of a microscope, and/or determining super-threshold particles with a coulter-counter.

Methods for coupling proteins and peptides to a solid phase matrix or carrier, the solid phase matrix materials useful in these methods, and means for elution, are well known to those of skill in the art.

The RPTP κ protein, or derivatives thereof having enzymatic activity, can be used for testing agents or compounds capable of enhancing or inhibiting the phosphatase activity. The ability of a compound under test to modify phosphatase activity can be tested in an in vitro system wherein the test compound is added to purified RPTP κ protein, or an enzymatically active derivative thereof, and the effects on enzyme activity measured using standard

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enzymological procedures well known to those of skill in the art.

Alternatively, the action of a compound on RPTP κ enzymatic activity can be measured in a whole cell preparation using live or fixed cells, or a membrane fraction derived from live or fixed cells. This method is useful for screening compounds acting via the extracellular receptor portion of the protein, as well as compounds acting directly on the enzymatic portion of the protein. A test compound is incubated with cells, or with a membrane preparation derived therefrom, which express high amounts of RPTP κ , such as transfected COS or NIH-3T3 cells. The amount of cellular phosphotyrosine is then measured, using methods well-known in the art (Honegger, A.M. et al., Cell 51:199-209 (1987); Margolis, B. et al., Cell 57:1101-1107 (1989)). The results are compared to results obtained in the absence of the test compound, or in the absence or presence of a known activator of RPTP κ enzymatic activity. In such studies, the action of the test compound in the presence of an activator of tyrosine kinase can also be measured. A compound which stimulates RPTP κ enzymatic activity will result in a net decrease in the amount of phosphotyrosine, whereas a compound which inhibits RPTP κ enzymatic activity will result in a net increase in the amount of phosphotyrosine. Compounds which inhibit homophilic Type II RPTP binding may also modulate the enzymatic activity of the RPTP molecules they affect, either by increasing or decreasing the RPTPs' phosphatase activity.

In the case of growth factor receptors which are tyrosine kinases, such as the receptors for epidermal growth factor (EGF) and for platelet-derived growth factor (PDGF), tyrosine phosphorylation is linked to

cell growth and to oncogenic transformation. Activation of a PTPase, leading to dephosphorylation, would serve as a counterregulatory mechanism to prevent or inhibit growth, and might serve as an endogenous regulatory mechanism against cancer. Thus, mutation or dysregulation of this receptor/enzyme system may promote susceptibility to cancer.

Inhibitory compounds which are found that are capable of inhibiting Type II RPTP homophilic binding may be used to modulate a variety of cellular processes including, but not limited to those involving cell-cell and/or cell-ECM interactions. Such processes include, but are not limited to normal cellular functions such as differentiation and cell cycle control; normal cellular behaviors including, but not limited to motility, contact inhibition, cell adhesion, and signal transduction; and abnormal or potentially deleterious processes such as cellular transformation to a cancerous state.

Inhibitory compounds which inhibit Type II RPTP homophilic binding may be used to modulate such processes in mammals by administration of an effective concentration of the inhibitory compound to a mammal, using techniques well known to those of ordinary skill in the art. Inhibitory compounds may include, but are not limited to, compounds comprising soluble RPTP Type II extracellular domains, for example, soluble RPTP κ extracellular domains.

Depending on the conditions being treated, agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition. Suitable routes may include oral, rectal, transmucosal, or intestinal administration;

parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, 5 intraperitoneal, intranasal, or intraocular injections, just to name a few. For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or 10 physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

The insulin receptor is also a tyrosine kinase, and phosphorylation of tyrosine in cells bearing 15 insulin receptors would be associated with normal physiological function. In contrast to the case of cell growth and cancer, activation of an RPTP would counteract insulin effects. Subnormal RPTP levels or enzymatic activity would act to remove a normal 20 counterregulatory mechanisms. Perhaps more important, though, over-activity, or inappropriate activation, of an RPTP, such as RPTP κ , would be expected to partially or totally inhibit the action of insulin on cells, leading to diabetes (of an insulin-resistant variety). 25 Thus, susceptibility to diabetes may be associated with RPTP κ dysregulation.

Therefore, the methods of the present invention for identifying normal or mutant genes encoding RPTP κ , 30 or for measuring the amount or activity of RPTP κ associated with a cell or tissue, can serve as methods for identifying susceptibility to cancer, diabetes, or other diseases associated with alterations in cellular phosphotyrosine metabolism.

The present invention provides methods for 35 evaluating the presence of, and the level of, normal

or mutant RPTPk in a cell or in a subject. Absence, or more typically, low expression of the RPTPk, or presence of a mutant RPTPk, in an individual may serve 5 as an important predictor of susceptibility to oncogenic transformation and the development of cancer. Alternatively, over-expression of RPTPk, possibly due to a mutant receptor/enzyme system 10 insensitive to negative regulation, or due to overabundance of a stimulatory ligand in the body, may serve as an important predictor of susceptibility to diabetes.

An oligonucleotide probe corresponding to a DNA 15 sequences encoding a part of RPTPk (see below) is used to test cells from a subject for the presence of DNA or RNA sequences encoding the RPTPk. A preferred probe would be one directed to the nucleic acid sequence 20 encoding at least 4 amino acid residues, and preferably at least 5 amino acid residues, of the RPTPk. Qualitative or quantitative assays can be 25 performed using such probes. For example, Northern analysis (see Section 7, below) is used to measure expression of an RPTPk mRNA in a cell or tissue preparation.

Such methods can be used even with very small 25 amounts of DNA obtained from an individual, following use of selective amplification techniques. Recombinant DNA methodologies capable of amplifying purified nucleic acid fragments have long been 30 recognized. Typically, such methodologies involve the introduction of the nucleic acid fragment into a DNA or RNA vector, the clonal amplification of the vector, and the recovery of the amplified nucleic acid 35 fragment. Examples of such methodologies are provided by Cohen et al. (U.S. Patent 4,237,224), Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, Second

Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), which references are herein incorporated by reference).

5 An in vitro enzymatic method which is capable of increasing the concentration of such desired nucleic acid molecules is called the "polymerase chain reaction or "PCR" (Mullis, K. et al., *Cold Spring Harbor Symp. Quant. Biol.* 51:263-273 (1986); Erlich, H. et al., EP 50424, EP 84796, EP 258017, EP 237362; 10 Mullis, K., EP 201184; Mullis, K. et al., US 4,683,202; Erlich, H., US 4,582,788; and Saiki, R. et al., US 4,683,194).

15 The PCR provides a method for selectively increasing the concentration of a particular nucleic acid sequence even when that sequence has not been previously purified and is present only in a single copy in a particular sample. The method can be used to amplify either single- or double-stranded DNA. The 20 method uses two oligonucleotide probes to serve as primers for the template-dependent, polymerase mediated replication of a desired nucleic acid molecule.

25 The precise nature of the two oligonucleotide probes of the PCR method is critical to the success of the method. Polymerase dependent amplification of a nucleic acid molecule proceeds by the addition of a 5' nucleotide triphosphate to the 3' hydroxyl end of a nucleic acid molecule. Thus, the action of a 30 polymerase extends the 3' end of a nucleic acid molecule. These inherent properties are exploited in the selection of the oligonucleotide probes of the PCR. The oligonucleotide sequences of the probes are selected such that they contain sequences identical to, or complementary to, sequences which flank the 35 particular nucleic acid sequence whose amplification

is desired. More specifically, the oligonucleotide sequence of the "first" probe is selected such that it is capable of hybridizing to an oligonucleotide sequence located 3' to the desired sequence, whereas 5 the oligonucleotide sequence of the "second" probe is selected such that it contains an oligonucleotide sequence identical to one present 5' to the desired region. Both probes possess 3' hydroxy groups, and 10 therefore can serve as primers for nucleic acid synthesis.

PCR reaction conditions are cycled between (a) those conducive to hybridization and nucleic acid polymerization, and (b) those which result in the 15 denaturation of duplex molecules. In the first step of the reaction, the nucleic acids of the sample are transiently heated, and then cooled, in order to denature any double-stranded molecules. The "first" and "second" probes are then added to the sample at a 20 concentration which greatly exceeds that of the desired nucleic acid molecule. Upon incubation under conditions conducive to hybridization and polymerization, the "first" probe will hybridize to the sample nucleic acid molecule at a position 3' to 25 the sequence to be amplified. If the nucleic acid molecule of the sample was initially double-stranded, the "second" probe will hybridize to the complementary strand of the nucleic acid molecule at a position 3' to the sequence which is the complement of the 30 sequence whose amplification is desired. Upon addition of a polymerase, the 3' ends of the "first" and (if the nucleic acid molecule was double-stranded) "second" probes will be extended. The extension of the "first" probe will result in the synthesis of an 35 oligonucleotide having the exact sequence of the desired nucleic acid. Extension of the "second" probe

will result in the synthesis of an oligonucleotide having the exact sequence of the complement of the desired nucleic acid.

5 The PCR reaction is capable of exponential amplification of specific nucleic acid sequences because the extension product of the "first" probe, of necessity, contains a sequence which is complementary to a sequence of the "second" probe, and thus can serve as a template for the production of an extension product of the "second" probe. Similarly, the extension product of the "second" probe, of necessity, contains a sequence which is complementary to a sequence of the "first" probe, and thus can serve as a template for the production of an extension product of the "first" probe. Thus, by permitting cycles of polymerization, and denaturation, a geometric increase in the concentration of the desired nucleic acid molecule can be achieved. For reviews of the PCR, 10 see: Mullis, K.B., *Cold Spring Harbor Symp. Quant. Biol.* 51:263-273 (1986); Saiki, R.K. et al. 15 *BioTechnology* 3:1008-1012 (1985); Mullis, K.B. et al. 20 *Meth. Enzymol.* 155:335-350 (1987).

25 In one embodiment, the present invention is directed to a naturally occurring mammalian RPTPK. In another embodiment, the present invention is directed to a recombinant mammalian RPTPK. The preferred mammalian RPTPK of the present invention is of human origin. The invention provides the naturally occurring molecule substantially free of other proteins with which it is natively associated. 30 "Substantially free of other proteins or glycoproteins" indicates that the protein has been purified away from at least 90 per cent (on a weight basis), and from even at least 99 per cent if desired, 35 of other proteins and glycoproteins with which it is

natively associated, and is therefore substantially free of them. That can be achieved by subjecting the cells, tissue or fluid containing the RPTP κ to 5 standard protein purification techniques such as an immunoabsorbent column bearing an antibody specific for the protein. Other forms of affinity purification utilize solid-phase substrates which bind the RPTP's enzymatic domain, or a ligand that will bind to the 10 receptor domain. Alternatively, the purification can be achieved by a combination of standard methods, such as ammonium sulfate precipitation, molecular sieve chromatography, and ion exchange chromatography.

It will be understood that the RPTP κ of the 15 present invention can be biochemically purified from a variety of cell or tissue sources. For preparation of naturally occurring RPTP κ , tissues such as mammalian brain, especially of human origin, are preferred.

Alternatively, because the gene for the RPTP κ 20 can be isolated or synthesized, the polypeptide can be synthesized substantially free of other mammalian proteins or glycoproteins in a prokaryotic organism or in a non-mammalian eukaryotic organism, if desired. As intended by the present invention, a recombinant 25 RPTP κ molecule produced in mammalian cells, such as transfected COS, NIH-3T3, or CHO cells, for example, is a protein with the naturally occurring amino acid sequence or is a functional derivative thereof. Where a naturally occurring protein or glycoprotein is 30 produced by recombinant means, it is provided substantially free of the other proteins and glycoproteins with which it is natively associated.

Alternatively, methods are well known for the 35 synthesis of polypeptides of desired sequence on solid phase supports and their subsequent separation from the support.

5 The present invention provides any of a number of "functional derivatives" of the RPTPk. By "functional derivative" is meant a "fragment," "variant," "analog," or "chemical derivative" of the RPTPk, which terms are defined below. A functional derivative retains at least a portion of the function of the RPTPk, such as (a) binding to a specific antibody, (b) 10 phosphatase enzymatic activity, or (c) binding of the extracellular "receptor" domain to a ligand, which permits its utility in accordance with the present invention.

15 A "fragment" of the RPTPk refers to any subset of the molecule, that is, a shorter peptide.

20 A "variant" of the RPTPk refers to a molecule substantially similar to either the entire peptide or a fragment thereof. Variant peptides may be conveniently prepared by direct chemical synthesis of the variant peptide, using methods well-known in the art.

25 Alternatively, amino acid sequence variants of the peptide can be prepared by mutations in the DNA which encodes the synthesized peptide. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence. Any combination of deletion, insertion, and substitution may also be made to arrive at the final construct, provided that the final construct possesses the desired activity. Obviously, the mutations that 30 will be made in the DNA encoding the variant peptide must not alter the reading frame and preferably will not create complementary regions that could produce secondary mRNA structure (see European Patent Publication EP 75444).

35 At the genetic level, these variants ordinarily are prepared by site-directed mutagenesis (as

exemplified by Adelman et al., DNA 2:183 (1983)) of nucleotides in the DNA encoding the protein or peptide molecule, thereby producing DNA encoding the variant, 5 and thereafter expressing the DNA in recombinant cell culture. The variants typically exhibit the same qualitative biological activity as the nonvariant protein or peptide.

An "analog" of the RPTP κ refers to a non-natural 10 molecule substantially similar to either the entire molecule or a fragment thereof.

A "chemical derivative" of the RPTP κ contains additional chemical moieties not normally a part of the peptide. Covalent modifications of the RPTP κ 15 protein or of a peptide derived therefrom, are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of 20 reacting with selected side chains or terminal residues.

Cysteinyl residues most commonly are reacted with alpha-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give 25 carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(5-imidazoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 30 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylprocarbonate, pH 5.5-7.0, because this 35 agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the

reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. 5 Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α -amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; 10 chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them 15 phenylglyoxal, 2,3- butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine 20 functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine ϵ -amino group.

The specific modification of tyrosyl residues per 25 se has been studied extensively, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizol and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

30 Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R'-N-C-N-R') such as 1-cyclohexyl-3-(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl 35 and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

5 Glutaminyl and asparaginyl residues may be deamidated to the corresponding glutamyl and aspartyl residues, under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

10 Derivatization with bifunctional agents is useful for cross-linking the protein or peptide to a water-insoluble support matrix or to other macromolecular carriers. Commonly used cross-linking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidyl-propionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light.

20 Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

25 Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the X-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, *PROTEINS: STRUCTURE AND MOLECULE PROPERTIES*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

30 Such derivatized moieties may improve the solubility, absorption, biological half life, and the

like. The moieties may alternatively eliminate or attenuate any undesirable side effect of the protein and the like. Moieties capable of mediating such effects are disclosed, for example, in REMINGTON'S PHARMACEUTICAL SCIENCES, 16th ed., Mack Publishing Co., Easton, PA (1980).

This invention is also directed to an antibody specific for an epitope of RPTPk, preferably, of human RPTPk, and the use of such an antibody to detect the presence of, or measure the quantity or concentration of, the RPTPk in a cell, a cell or tissue extract, or a biological fluid.

The term "antibody" is meant to include polyclonal antibodies, monoclonal antibodies (mAbs), chimeric antibodies, and anti-idiotypic (anti-Id) antibodies.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, preferably the RPTPk protein or glycoprotein, a peptide derived therefrom or an epitope thereof.

Monoclonal antibodies are a substantially homogeneous population of antibodies to specific antigens. MAbs may be obtained by methods known to those skilled in the art. See, for example Kohler and Milstein, *Nature* 256:495-497 (1975) and U.S. Patent No. 4,376,110. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, and any subclass thereof. The hybridoma producing the mAbs of this invention may be cultivated *in vitro* or *in vivo*. Production of high titers of mAbs *in vivo* production makes this the presently preferred method of production. Briefly, cells from the individual hybridomas are injected intraperitoneally into pristane-primed BALB/c mice to produce ascites fluid.

containing high concentrations of the desired mAbs. MAbs of isotype IgM or IgG may be purified from such ascites fluids, or from culture supernatants, using 5 column chromatography methods well known to those of skill in the art.

Chimeric antibodies are molecules different portions of which are derived from different animal species, such as those having variable region derived 10 from a murine mAb and a human immunoglobulin constant region. Chimeric antibodies and methods for their production are well-known in the art (Cabilly et al., Proc. Natl. Acad. Sci. USA 81:3273-3277 (1984); Morrison et al., Proc. Natl. Acad. Sci. USA 81:6851- 15 6855 (1984); Boulianne et al., Nature 312:643-646 (1984); Neuberger et al., Nature 314:268-270 (1985); Taniguchi et al., European Patent Publication EP171496 (February 19, 1985); Morrison et al., European Patent Publication EP 173494 (March 5, 1986); Neuberger et 20 al., PCT Publication WO 86/01533 (March 13, 1986); Kudo et al., European Patent Publication EP 184187 (June 11, 1986); Sahagan et al., J. Immunol. 137:1066- 1074 (1986); Robinson et al., International Patent 25 Publication #PCT/US86/02269 (7 May 1987); Liu et al., Proc. Natl. Acad. Sci. USA 84:3439-3443 (1987); Sun et al., Proc. Natl. Acad. Sci. USA 84:214-218 (1987); Better et al., Science 240:1041-1043 (1988)). These 30 references are hereby incorporated by reference.

An anti-idiotypic (anti-Id) antibody is an 30 antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An anti-Id antibody can be prepared by immunizing an animal of the same species and genetic type (e.g. mouse strain) as the source of the mAb with 35 the mAb to which an anti-Id is being prepared. The immunized animal will recognize and respond to the

5 idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants (the anti-Id antibody). The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be 10 epitopically identical to the original mAb which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other hybrid clones expressing antibodies of identical specificity.

15 Accordingly, mAbs generated against RPTP κ may be used to induce anti-Id antibodies in suitable animals, such as BALB/c mice. Spleen cells from such immunized mice are used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c mice. 20 Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for an RPTP κ epitope. The anti-Id mAbs thus have their own idiotypic epitopes, or "idiotypes" structurally similar to the epitope being evaluated, such as an epitope of RPTP κ .

25 The term "antibody" is also meant to include both intact molecules as well as fragments thereof, such as, for example, Fab and F(ab')₂, which are capable of binding antigen. Fab and F(ab')₂ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl et al., J. Nucl. Med. 24:316-325 (1983)).

30 It will be appreciated that Fab and F(ab')₂, and other fragments of the antibodies useful in the present invention may be used for the detection and

quantitation of RPTP κ according to the methods disclosed herein for intact antibody molecules. Such fragments are typically produced by proteolytic 5 cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab') $_2$ fragments).

An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the 10 antibody. The term "epitope" is meant to refer to that portion of any molecule capable of being bound by an antibody which can also be recognized by that antibody. Epitopes or "antigenic determinants" usually consist of chemically active surface groupings of 15 molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics. An "antigen" is a molecule or a portion of a molecule capable of being bound by an 20 antibody which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen may have one, or more than one epitope.

An antibody is said to be specific for an antigen 25 because it reacts in a highly selective manner, with that antigen and not with the multitude of other antigens which are structurally distinct.

The antibodies or antibody fragments of the present invention may be used to quantitatively or 30 qualitatively detect the presence of cells which express the RPTP κ protein. This can be accomplished by immunofluorescence techniques employing a fluorescently labeled antibody (see below) coupled with light microscopic, flow cytometric, or 35 fluorimetric detection. For such methods, the

antibody is preferably specific for an extracellular epitope of RPTPk.

The antibodies (or fragments thereof) useful in the present invention may be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of RPTPk. *In situ* detection may be accomplished by removing a histological specimen from a subject, and providing a labeled antibody or antibody fragment of the present invention to such a specimen, preferably by applying or overlaying the antibody over the specimen. Through the use of such a procedure, it is possible to determine not only the presence of RPTPk but also its distribution in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection. Such assays for RPTPk typically comprise incubating a biological sample, such as a biological fluid, a tissue extract, freshly harvested cells, or cells which have been incubated in tissue culture, in the presence of a detectably labeled antibody specific for RPTPk, and detecting the antibody by any of a number of techniques well-known in the art.

The biological sample may be incubated with a solid phase support or carrier such as nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled RPTPk-specific antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound

label on said solid support may then be detected by conventional means.

By "solid phase support" is intended any support capable of binding antigen or antibodies. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, and magnetite. The preferred carrier is totally insoluble in the solution in which the assay of the present invention takes place; partially soluble carriers well-known in the art may also be used. The support material may have virtually any possible structural configuration so long as the support-coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of anti-RPTP κ antibody may be determined according to well-known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

One of the ways in which the RPTP κ -specific antibody can be detectably labeled is by linking the antibody, or a second antibody which binds to the anti-RPTP κ antibody, to an enzyme and use in an enzyme immunoassay (EIA). This enzyme, in turn, when later exposed to an appropriate substrate, will react with

the substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means.

5 Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be
10 accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

15 20 Detection may be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect RPTP κ through the use of a radioimmunoassay (RIA) (see, for example, Work, T.S. et al., *LABORATORY TECHNIQUES AND BIOCHEMISTRY IN MOLECULAR BIOLOGY*, North Holland Publishing Company, New York, 1978, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a
25 30 gamma counter or a scintillation counter or by autoradiography.

35 It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used

fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, α - phthaldehyde and fluorescamine.

5 The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu , or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or 10 ethylenediaminetetraacetic acid (EDTA).

15 The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a 20 chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

25 Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

30 The antibody molecules of the present invention may be adapted for utilization in an immunometric assay, also known as a "two-site" or "sandwich" assay. In a typical immunometric assay, a quantity of unlabeled antibody (or fragment of antibody) is bound to a solid support and a quantity of detectably 35 labeled soluble antibody is added to permit detection and/or quantitation of the ternary complex formed

between solid-phase antibody, antigen, and labeled antibody.

Typical, and preferred, immunometric assays 5 include "forward" assays in which the antibody bound to the solid phase is first contacted with the sample being tested to extract the antigen from the sample by formation of a binary solid phase antibody-antigen complex. After a suitable incubation period, the 10 solid support is washed to remove the residue of the fluid sample, including unreacted antigen, if any, and then contacted with the solution containing a labeled second antibody (which functions as a "reporter molecule"). After a second incubation period to 15 permit the labeled antibody to complex with the antigen bound to the solid support through the unlabeled antibody, the solid support is washed a second time to remove the unreacted labeled antibody.

In another type of "sandwich" assay, which may 20 also be useful with the antigens of the present invention, the so-called "simultaneous" and "reverse" assays are used. A simultaneous assay involves a single incubation step as the antibody bound to the solid support and labeled antibody are both added to 25 the sample being tested at the same time. After the incubation is completed, the solid support is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the solid support is then determined 30 as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first 35 of a solution of labeled antibody to a fluid sample followed by the addition of unlabeled antibody bound to a solid support after a suitable incubation period is utilized. After a second incubation, the solid

phase is washed in conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labeled antibody. The 5 determination of labeled antibody associated with a solid support is then determined as in the "simultaneous" and "forward" assays.

The presence of normally functioning RPTP κ in a subject can also be tested using direct enzymatic 10 assays, for the tyrosine phosphatase activity. Such biochemical measurements can be performed *in vitro*, using purified enzymes, allowing precise measurements of enzyme activity, or with membrane preparations, or whole cells, where the net phosphotyrosine level is 15 determined.

In additional embodiments of the present invention, a nucleic acid molecule, preferably DNA, comprising a sequence encoding an RPTP κ protein and methods for expressing the DNA molecule are provided. 20 One of ordinary skill in the art will know how to identify and clone additional RPTP molecules, of human or other mammalian species, which have sequence homology to the RPTP κ molecules described herein, using the genetic sequences and oligonucleotides of 25 the present invention without undue experimentation. Furthermore, manipulation of the genetic constructs of the present invention allow the grafting of a particular ligand-binding receptor domain onto the transmembrane and catalytic portions of the RPTP κ resulting in chimeric molecules. Non-limiting 30 examples of such chimeric molecules include RPTP κ wherein the receptor portion is an epidermal growth factor receptor, a fibroblast growth factor receptor, and the like. Genetically engineered chimeric receptors are known in the art (see, for example, Riedel, 35 H. et al., *Nature* 324:628-670 (1986)).

Genetic constructs encoding RPTPK, functional derivative thereof, and chimeric molecules such as those described above, can be used in gene therapy.

5 An abnormal or dysfunctional RPTPK, which results in disease, may be replaced by infusion or implantation of cells of the desired lineage (such as hemopoietic cells, neurons, etc.) transfected with DNA encoding normal RPTPK. Alternatively, or additionally, cells

10 carrying a chimeric RPTPK having a receptor portion which binds a ligand of choice (e.g., EGF) can be used for such gene therapy.

The recombinant DNA molecules of the present invention can be produced through any of a variety of means, such as, for example, DNA or RNA synthesis, or more preferably, by application of recombinant DNA techniques. Techniques for synthesizing such molecules are disclosed by, for example, Wu, R., et al. (Prog. Nucl. Acid. Res. Molec. Biol. 21:101-141 15 (1978)), and procedures for constructing recombinant molecules can be found in Sambrook et al. (supra).

Oligonucleotides representing a portion of an RPTPK are useful for screening for the presence of genes encoding such proteins and for the cloning of an 20 RPTPK gene. Techniques for synthesizing such oligonucleotides are disclosed by, for example, Wu, R., et al., supra.

Protein molecules are fragmented as with cyanogen bromide, or with proteases such as papain, 25 chymotrypsin, trypsin, etc. (Oike, Y., et al., J. Biol. Chem. 257:9751-9758 (1982); Liu, C., et al., Int. J. Pept. Protein Res. 21:209-215 (1983)). Because the genetic code is degenerate, more than one codon 30 may be used to encode a particular amino acid (Watson, J.D., In: MOLECULAR BIOLOGY OF THE GENE, 4th Ed., 35 Benjamin/Cummings Publishing Co., Inc., Menlo Park, CA

(1987)). Using the genetic code, one or more different oligonucleotides can be identified, each of which would be capable of encoding the amino acid.

5 The probability that a particular oligonucleotide will, in fact, constitute the actual XXX-encoding sequence can be estimated by considering abnormal base pairing relationships and the frequency with which a particular codon is actually used (to encode a 10 particular amino acid) in eukaryotic cells. Such "codon usage rules" are disclosed by Lathe, R., et al., *J. Mol. Biol.* 183:1-12 (1985). Using such "codon usage rules", a single oligonucleotide, or a set of oligonucleotides, that contains a theoretical "most 15 probable" nucleotide sequence capable of encoding RPTPK is identified.

Although occasionally an amino acid sequence may be encoded by only a single oligonucleotide, frequently the amino acid sequence may be encoded by 20 any of a set of similar oligonucleotides. Importantly, whereas all of the members of this set contain oligonucleotides which are capable of encoding the peptide fragment and, thus, potentially contain the same oligonucleotide sequence as the gene which 25 encodes the peptide fragment, only one member of the set contains the nucleotide sequence that is identical to the nucleotide sequence of the gene. Because this member is present within the set, and is capable of hybridizing to DNA even in the presence of the other 30 members of the set, it is possible to employ the unfractionated set of oligonucleotides in the same manner in which one would employ a single oligonucleotide to clone the gene that encodes RPTPK.

The oligonucleotide, or set of oligonucleotides, 35 containing the theoretical "most probable" sequence capable of encoding the RPTPK fragment is used to

identify the sequence of a complementary oligonucleotide or set of oligonucleotides which is capable of hybridizing to the "most probable" sequence, or set of sequences. An oligonucleotide containing such a complementary sequence can be employed as a probe to identify and isolate the RPTPk gene (Sambrook et al., *supra*).

A suitable oligonucleotide, or set of oligonucleotides, capable of encoding a fragment of the RPTPk gene (or complementary to such an oligonucleotide) is identified as above and synthesized, using procedures well known in the art (Belagaje, R., et al., *J. Biol. Chem.* 254:5765-5780 (1979); Maniatis, T., et al., In: *MOLECULAR MECHANISMS IN THE CONTROL OF GENE EXPRESSION*, Nierlich, D.P., et al., Eds., Acad. Press, NY (1976); Wu, R., et al., *Prog. Nucl. Acid Res. Molec. Biol.* 21:101-141 (1978); Khorana, R.G., *Science* 203:614-625 (1979)). DNA synthesis may be achieved using an automated synthesizers. The oligonucleotide probe or set is hybridized by means well known in the art, against a DNA or, more preferably, a cDNA preparation derived from cells which are capable of expressing the RPTPk gene. Techniques of nucleic acid hybridization are disclosed by Sambrook et al. (*supra*), and by Haymes, B.D., et al. (In: *NUCLEIC ACID HYBRIDIZATION, A PRACTICAL APPROACH*, IRL Press, Washington, DC (1985)), which references are herein incorporated by reference. Techniques such as, or similar to, those described above have successfully enabled the cloning of genes for human aldehyde dehydrogenases (Hsu, L.C. et al., *Proc. Natl. Acad. Sci. USA* 82:3771-3775 (1985)), fibronectin (Suzuki, S., et al., *EMBO J.* 4:2519-2524 (1985)), the human estrogen receptor gene (Walter, P., et al., *Proc. Natl. Acad. Sci. USA*

82:7889-7893 (1985)), tissue-type plasminogen activator (Pennica, D., et al., *Nature* 301:214-221 (1983)) and human term placental alkaline phosphatase complementary DNA (Kam, W., et al., *Proc. Natl. Acad. Sci. USA* 82:(715-8719 (1985)).

In an alternative way of cloning the RPTP κ gene, a library of expression vectors is prepared by cloning DNA or, more preferably, cDNA (from a cell capable of expressing RPTP κ) into an expression vector. The library is then screened for members capable of expressing a protein which binds to an anti-RPTP κ antibody, and which has a nucleotide sequence that is capable of encoding a polypeptide that has the same amino acid sequence as all or part of RPTP κ . In this embodiment, DNA, or more preferably cDNA, is extracted and purified from a cell which is capable of expressing RPTP κ protein. The purified cDNA is fragmented (by shearing, endonuclease digestion, etc.) to produce a pool of DNA or cDNA fragments. DNA or cDNA fragments from this pool are then cloned into an expression vector in order to produce a genomic or cDNA library of expression vectors whose members each contain a unique cloned DNA or cDNA fragment.

An "expression vector" is a vector which (due to the presence of appropriate transcriptional and/or translational control sequences) is capable of expressing a DNA molecule which has been cloned into the vector and of thereby producing a peptide or protein. Expression of the cloned sequences occurs when the expression vector is introduced into an appropriate host cell. If a prokaryotic expression vector is employed, then the appropriate host cell would be any prokaryotic cell capable of expressing the cloned sequences. If a eukaryotic expression vector is employed, then the appropriate host cell

would be any eukaryotic cell capable of expressing the cloned sequences. Importantly, since eukaryotic DNA may contain intervening sequences, and since such sequences cannot be correctly processed in prokaryotic cells, it is preferable to employ cDNA from a cell which is capable of expressing RPTPk in order to produce a prokaryotic genomic expression vector library. Procedures for preparing cDNA and for producing a genomic library are disclosed by Sambrook et al. (supra).

A DNA sequence encoding RPTPk of the present invention, or encoding functional derivatives thereof, may be recombined with vector DNA in accordance with conventional techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Techniques for such manipulations are disclosed by Sambrook et al., supra, and are well known in the art.

A nucleic acid molecule, such as DNA, is "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are "operably linked" to a polypeptide coding sequence. An operable linkage is a linkage in which the regulatory DNA sequences and the coding sequence are connected in such a way as to permit gene expression. The precise nature of the regulatory regions needed for gene expression may vary from organism to organism, but shall in general include a promoter region which, in prokaryotes, contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which,

when transcribed into RNA, will signal the initiation of protein synthesis. Such regions will normally include those 5'-non-coding sequences involved with 5 initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like.

If desired, the non-coding region 3' to the coding sequence may be obtained by the above-described 10 methods. This region may be retained for its transcriptional termination regulatory sequences, such as termination and polyadenylation. Thus, by retaining the 3'-region naturally contiguous to the DNA coding sequence, the transcriptional termination 15 signals may be provided. Where the transcriptional termination signals are not satisfactorily functional in the host cell used to express the protein, then a 3' region functional in that host cell may be substituted.

Two DNA sequences (such as a promoter region 20 sequence and a RPTP κ coding sequence) are said to be operably linked if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere 25 with the ability of the promoter to regulate the transcription of the RPTP κ coding sequence. A promoter region is operably linked to a DNA coding sequence if the promoter is capable of effecting transcription of the coding sequence. Thus, to 30 express the protein, transcriptional and translational signals recognized by an appropriate host are necessary. In order to be "operably linked" it is not necessary that two sequences be immediately adjacent to one another.

35 A promoter is a double-stranded DNA (or RNA) molecule which is capable of binding to RNA polymerase

and promoting the transcription of an "operably linked" nucleic acid coding sequence. As used herein, a "promoter sequence" is the sequence of the promoter which is found on that strand of the DNA (or RNA) which is transcribed by the RNA polymerase. A "promoter sequence complement" has a sequence which is the complement of the "promoter sequence." Hence, upon extension of a primer DNA or RNA adjacent to a single-stranded "promoter sequence complement" or, of a "promoter sequence," a double-stranded molecule is created which will contain a functional promoter, if that extension proceeds towards the "promoter sequence" or the "promoter sequence complement." This functional promoter will direct the transcription of a nucleic acid molecule which is operably linked to that strand of the double-stranded molecule which contains the "promoter sequence" (and not that strand of the molecule which contains the "promoter sequence complement").

Certain RNA polymerases exhibit a high specificity for such promoters. The RNA polymerases of the bacteriophages T7, T3, and SP-6 are especially well characterized, and exhibit high promoter specificity. The promoter sequences which are specific for each of these RNA polymerases also direct the polymerase to transcribe from only one strand of a duplex DNA template. Strand selection is determined by the orientation of the promoter sequence, and determines the direction of transcription since RNA is only polymerized enzymatically by the addition of a nucleotide 5' phosphate to a 3' hydroxyl terminus.

The promoter sequences of the present invention may be either prokaryotic, eukaryotic or viral. Suitable promoters are repressible, or, more preferably, constitutive. Examples of suitable

prokaryotic promoters include promoters capable of recognizing the T4 (Malik, S. et al., *J. Biol. Chem.* 263:1174-1181 (1984); Rosenberg, A.H. et al., *Gene* 59:191-200 (1987); Shinedling, S. et al., *J. Molec. Biol.* 195:471-480 (1987); Hu, M. et al., *Gene* 42:21-30 (1986)), T3, Sp6, and T7 (Chamberlin, M. et al., *Nature* 228:227-231 (1970); Bailey, J.N. et al., *Proc. Natl. Acad. Sci. USA* 80:2814-2818 (1983); Davanloo, P. et al., *Proc. Natl. Acad. Sci. USA* 81:2035-2039 (1984)) polymerases; the P_R and P_L promoters of bacteriophage λ (*THE BACTERIOPHAGE LAMBDA*, Hershey, A.D., Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY (1973); *Lambda II*, Hendrix, R.W., Ed., Cold Spring Harbor Press, Cold Spring Harbor, NY (1980)); the *trp*, *recA*, heat shock, and *lacZ* promoters of *E. coli*; the α -amylase (Ulmanen, I., et al., *J. Bacteriol.* 162:176-182 (1985)) and the σ -28-specific promoters of *B. subtilis* (Gilman, M.Z., et al., *Gene* 32:11-20 (1984)); the promoters of the bacteriophages of *Bacillus* (Gryczan, T.J., In: *THE MOLECULAR BIOLOGY OF THE BACILLI*, Academic Press, Inc., NY (1982)); *Streptomyces* promoters (Ward, J.M., et al., *Mol. Gen. Genet.* 203:468-478 (1986)); the *int* promoter of bacteriophage λ ; the *bla* promoter of the β -lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene of pPR325, etc. Prokaryotic promoters are reviewed by Glick, B.R., *J. Ind. Microbiol.* 1:277-282 (1987)); Cenatiempo, Y. (*Biochimie* 68:505-516 (1986)); Watson, J.D. et al. (*supra*); and Gottesman, S. *Ann. Rev. Genet.* 18:415-442 (1984)). Preferred eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer, D., et al., *J. Mol. Appl. Gen.* 1:273-288 (1982)); the TK promoter of Herpes virus (McKnight, S., *Cell*

31:355-365 (1982)); the SV40 early promoter (Benoist, C., et al., *Nature* 290:304-310 (1981)); and the yeast *gal4* gene promoter (Johnston, S.A., et al., *Proc. Natl. Acad. Sci. USA* 79:6971-6975 (1982); Silver, P.A., et al., *Proc. Natl. Acad. Sci. USA* 81:5951-5955 (1984)). All of the above listed references are incorporated by reference herein.

Strong promoters are preferred. Examples of such preferred promoters are those which recognize the T3, SP6 and T7 polymerases, the P_L promoter of bacteriophage λ , the *recA* promoter and the promoter of the mouse metallothionein I gene. A most preferred promoter for eukaryotic expression of RPTP κ is an SV40 promoter such as that driving transcription in the pLSV vector (Livneh, E., et al., (1986) *J. Biol. Chem.* 261:12490- 12497). The sequences of such polymerase recognition sites are disclosed by Watson, J.D. et al. (*supra*).

Having now generally described the invention, the same will be more readily understood through reference to the following example which is provided by way of illustration, and is not intended to be limiting of the present invention, unless specified.

25

6. EXAMPLE: ISOLATION AND ANALYSIS OF MURINE RPTP κ cDNA CLONES

In an effort to identify new PTPases, a mouse brain cDNA library in λ gt11 was screened under relaxed 30 stringency conditions using as a probe an oligonucleotide corresponding to the intracellular two tandem PTPase homology domains of human CD45 (Sap et al., *supra*). Following initial characterization and classification of the isolated clones, several 35 subsequent rounds of screening mouse brain libraries at high stringency yielded a set of cDNA fragments

that together encompassed the entire coding sequence for RPTPk. The relationship between the different RPTPk cDNA clones isolated was confirmed by Northern and reverse transcriptase/PCR analyses (see Materials and Methods section and FIG. 2 for details).

6.1. LIBRARY SCREENING

10 The original RPTPk cDNA clone was isolated by low-stringency screening of a λgt11 mouse brain cDNA library with a probe consisting of the intracellular domain of human CD45, which contains two tandem PTPase domains (see: Sap, J. et al., 1990 Proc. Natl. Acad. Sci. USA 87:6112-6116, for details). After initial characterization, one of the isolated clones (λ-50, containing a 935 nucleotide fragment with characteristic homology to members of the PTPase family), was used to rescreen the same library, yielding clones λ-602 and λ-604. Sequence analysis showed that clone λ-602 was identical to λ-604 at both extremities, but was interrupted by a sequence containing stop codons in all three reading frames. Its analysis was therefore discontinued, since it is likely to represent an incompletely spliced RNA species. By contrast, λ-604 appeared to contain one PTPase homology domain and an additional 2042 nt. of upstream coding sequence, including a likely membrane-spanning region.

30 In order to obtain a full length RPTPk cDNA, the entire insert of clone 604 was used to screen another (randomly primed) mouse brain cDNA library (Clontech), leading to the isolation of two hybridizing clones, λ-35 and λ-37. Clone 35 appeared to overlap with the N-terminus of clone 604 and to encompass the translational initiation codon for the RPTPk precursor

protein (see results section). Initial sequence analysis of clone 37 however revealed no overlap with the clone 604 probe, although it contained a clear additional PTPase homology followed by a stop codon in a position characteristic for the second PTPase domain of a RPTPase. Several controls were used to show that clone 37 corresponds to the bona fide C-terminus of RPTP κ . In Northern analysis, clones 37 and 604 recognize identical mRNA species in all mouse tissues examined.

10 A reverse transcriptase/PCR analysis on mouse liver poly(A)+ RNA using primers corresponding to clones 604 and 37, followed by cloning and sequencing, 15 yielded a fragment of the expected size, exactly joining both clones at the same EcoRI site where each isolated cDNA clone ended.

10 In retrospect, clone 37 was therefore most likely picked up in the screening with the clone 604 fragment due to the existence of an additional small cDNA 20 fragment in the original λ -37 phage isolate that went undetected due to its small size, or by fortuitous crosshybridization between the two PTPase homologies of RPTP κ . A schematic summary of the different cDNA 25 clones discussed is included in FIG. 2.

6.2. NUCLEOTIDE SEQUENCE DETERMINATION

30 cDNA fragments were isolated from phage clones, subcloned into Bluescript cloning vectors and subjected to sequence analysis by the 35 dideoxynucleotide chain termination method (Sequenase, United States Biochemical) using synthetic oligonucleotide primers. All sequences were determined on both strands. Sequences were assembled and analyzed using the GCG 7 software package

(Devereux, J. et al., 1984 *Nuc. Acids Res.* 12:387-395). The assembled RPTP κ cDNA nucleotide sequence was submitted to Genbank under accession number
5 L10106.

6.3. SEQUENCE ALIGNMENTS

All DNA and protein data base searches were done with the Genetic Computer Group sequence analysis 10 software package (Devereux et al., *Nucleic Acid Res.* 12:387-396 (1989)). The SwissProt and Gene Bank\European Molecular Biology Laboratory data bases were searched with FASTA and TFASTA, respectively 15 (Pearson and Lipman, *Proc. Natl. Acad. Sci.* 85:2444-2448 (1988)). Proteins were aligned with the Genetics Computer Group programs, LINEUP, PILEUP, PRETTY and BESTFIT.

20 6.4. RESULTS AND DISCUSSION

6.4.1. ISOLATION AND SEQUENCE ANALYSIS OF cDNA CLONES ENCODING MURINE RPTP κ

The nucleotide sequence of murine RPTP κ (SEQ ID 25 NO:3) is shown in FIG. 1(1) - 1(5). The complete amino acid sequences of RPTP κ (SEQ ID NO:1) is shown in FIG. 1(1) through 1(5) and in FIG. 3.

The assembled RPTP κ cDNA sequence can be divided into a 5' untranslated region of 1072 base pairs, a 30 single open reading frame of 4374 base pairs and a 3' untranslated region of 388 base pairs. The deduced amino acid sequence of the RPTP κ precursor protein is shown in FIG. 3. The translational initiation codon is identified by a standard environment for initiation 35 of translation (Kozak, *supra*) and by the existence of an upstream in-frame stop codon (position -252), and

- 60 -

is followed by a hydrophobic region that may serve as a signal peptide. A second hydrophobic region is found between amino acid residues 753 and 774 and is followed by a series of predominantly basic residues, 5 characteristic of a stop transfer sequence. These features delineate a putative extracellular region of 752 amino acid residues (including the signal sequence), and an intracellular portion of 683 amino acids. 10 The latter contains the tandem repeat of two PTPase homologies typical for most RPTPases isolated so far (Fischer, E.H. et al., 1991 *Science* 253:401-406).

An intriguing feature of RPTP κ is the extended 15 distance between its trans-membrane segment and the start of the first phosphatase homology domain. This region is about 70 residues longer than in all other previously described RPTPases, with the exception of mRPTP μ (Gebbink et al., *supra*).

Interestingly, a variant of RPTP κ was found by 20 the present inventors' laboratory to contain a similarly-sized insertion in the same position. It is conceivable that such an insertion generated by alternative splicing might constitute a separate functional unit in RPTPases.

The first approximately 170 amino acids of RPTP κ show similarity (26% overall identity) to a region in the *Xenopus* cell surface protein A5 with features of Ig-like domains (FIG. 5). The A5 protein is thought 25 to function in recognition between input and target neurons in the visual system (Takagi, S. et al., 1991 *Neuron* 7:295-307).

This first domain is followed by one Ig-like repeat (approximately residues 210 to 270) and four 30 putative fibronectin type III-like (FN-III) repeats (residues 296 to 681). Database searching revealed 35

clear similarity of these FN-III domains to similar domains in the tyrosine phosphatases R-PTP μ and LAR, the *Drosophila* R-PTPases DLAR and DPTP10D, and 5 *Drosophila* neuroglian (Bieber, A.J. et al. 1989. *Cell* 59:447-460; Gebbink et al., *supra*; Streuli, M. et al., 1988, *supra*; Streuli, M. et al., 1989, *supra*; Tian et al., *supra*; Yang et al., *supra*).

Some other features of the extracellular domain 10 of RPTP κ are noteworthy. First, it contains the sequence HAV (amino acids 340-342; within the first FN-III repeat) implicated in cell-cell contact in members of the cadherin family (Blaschuk, O.W. et al., 1990 *J.Mol.Biol.* 211:679-682). Second, the 15 extracellular domain (640-643) contains the sequence RTKR, a consensus cleavage site for the processing endoprotease furin (Hosaka, M. et al., 1991 *J. Biol.Chem.* 266:12127-12130). Other potential posttranslational modification sites include 12 20 potential N-linked glycosylation sites, and SG-motifs which are candidates for chondroitin sulfate attachment (residues 172, 176, 277, 333, 662) (Kjellen, L. et al. 1991 *Annu. Rev. Biochem.* 60:443-470).

Overall, the sequence of RPTP κ shows a high 25 degree of sequence similarity to mRPTP μ (77% overall similarity at the amino acid level) (Gebbink et al., *supra*). The sequence identity between this pair of related R-PTPases is highest in the first PTPase 30 homology domain (80% as compared to 74% identity for the second PTPase domain). This is in contrast to the situation that has been observed for the relationship between the closely related pairs of R-PTPases LAR and HPTP δ , and RPTP β /HPTP γ and RPTP γ (Kaplan, R. et al. 35 1990 *Proc. Natl. Acad. Sci. USA* 87:7000-7004; Krueger, N.X. et al., 1990 *EMBO J.* 9:3241-3252; Streuli, M. et

al., 1988, *supra*). The latter pairs of related R-PTPases are more related in their second PTPase homology domains. The sequence of RPTP κ is also highly similar to that of PCR fragment PTP 191-33 described by Nishi, M. et al., 1990 *FEBS Lett.* 271:178-180.

7. EXAMPLE: EXPRESSION AND TISSUE DISTRIBUTION OF RPTP κ

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7.1. TISSUE EXPRESSION AND NORTHERN ANALYSIS

Poly(A)+RNA was isolated from adult mouse tissues by oligo(dT) selection as described previously (Vennstrom, B. et al. 1982 *Cell* 28:135-143). Five μ g of poly(A)+ RNA per lane were fractionated on formaldehyde-containing 1% agarose gels, transferred to Nytran membranes, and probed under high stringency conditions with different regions of the RPTP κ cDNA. RNA loading and quality was controlled for by ethidium bromide staining.

7.1.1. EXPRESSION OF THE RPTP κ PROTEIN

25 In order to assemble a full-length RPTP κ cDNA from the various isolated fragments, a convenient fragment which included the N-terminus was generated from clone 35 by a PCR reaction using the N terminal primer 5'GAGCCGGCTCGAGTTAACGCCATGGATGTGGCGGCCG3' (SEQ ID NO:5) and the C-terminal primer 5'GCTCACAGCTAGTTCAGCCC3' (SEQ ID NO:6). This manipulation also removed all of the 5'untranslated sequences, while retaining an optimized Kozak consensus sequence for translation initiation (Kozak, 30 M. 1983 *Microbiol. Rev.* 47:1-45).

The amplified 470 nucleotide product was digested with Sac II and PpuM 1, and cloned between the Sac II and PpuM I sites of clone 604, yielding plasmid pK₀ 5 (the Sac II site being in the polylinker region of the Bluescript cloning vector). The 1.1 kb Eco RI fragment from clone 37 (containing the C-terminal end of the coding sequence) was then cloned into the unique and corresponding Eco RI site of pK₀ in the 10 appropriate orientation, yielding construct pK₁ containing the fully assembled coding sequence without the 5' untranslated sequences. The modified cDNA was then released as one fragment using Hpa I (N-terminal) and Xho I (C-terminal), and cloned between the Sma I 15 and Sal I sites of a CMV-enhancer/promoter-driven eukaryotic expression vector.

7.1.2. GENERATION OF ANTISERA SPECIFIC FOR EPITOPEs OF RPTP κ

20 Antigenicity of peptides included in the RPTP κ protein was predicted using the Jameson-Wolf algorithm included in the GCG 7 Peptidestructure program (Devereux, J. et al., 1984 *Nucl. Acids Res.* 12:387-395). Two peptides were synthesized. The 25 peptides were coupled to the protein keyhole limpet hemocyanin by glutaraldehyde crosslinking and injected into rabbits at two week intervals (100 pg per injection).

30 The first peptide corresponded to a site near the predicted N-terminus of the RPTP κ protein (SEQ ID NO:1), specifically, residues 60-76, having the sequence SAQEPHYLLPPEMPQGST. Immunization with this peptide yielded antiserum 116.

35 The second peptide corresponded to a region located at the N-terminus of the first PTPase homology in the intracellular region of the RPTP κ protein (SEQ

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ID NO:1), specifically, residues 910 to 929 having the sequence SASWDVAKKDQNRAK. Immunization with this peptide yielded antiserum 122) (FIG. 14).

5

7.1.3. TRANSFECTION, LABELING AND
IMMUNOPRECIPITATION

Subconfluent cultures of COS or HeLa cells in 10 cm diameter dishes (as indicated) were transfected by 10 the DEAE-dextran or calcium phosphate technique, respectively. Between 48 and 72 hours after transfection, the cells were metabolically labeled for 15 2 hours in methionine-free medium containing 50 μ Ci/ml 35 S-methionine (ICN). In the pulse-chase analysis 20 shown in FIG. 10, cells were labeled with 200 μ Ci/ml of the isotope. After labeling, cells were washed in PBS and lysed in Triton-X-100 lysis buffer (50 mM Hepes pH 7.5, 150 μ M NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton-X-100, 200 μ g/ml PMSF, 10 μ g/ml Aprotinin, 10 μ g/ml Leupeptin) at 4°C.

Cell lysates were incubated at 4°C for 2 hours with Protein A-Sepharose previously preincubated with the respective anti-RPTP κ antibody. Where indicated, 25 20 μ g of the antigenic peptide was included in the immunoprecipitation reaction as a control for specificity. Immunoprecipitates were washed with high, medium and low salt buffers (Lev, S. et al., 1991 EMBO J. 10:647-654), with the exception of the 30 experiment depicted in FIG. 12 where washing was with HNTG-buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton-X-100). Immunoblotting analyses were performed using standard procedures.

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7.1.4. PROTEIN TYROSINE PHOSPHATASE
ENZYMATIC ASSAY

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Phosphatase enzymatic assays were performed with RPTP κ protein immunoprecipitated with antiserum 116 (specific for the extracellular domain) from 5 transiently transfected COS cells. The protein A-Sepharose/RPTP κ immunoprecipitated complexes were washed 4 times with HNTG, and once with M7.6 buffer (60 mM Tris, pH 7.6, 5 mM EDTA, 10 mM DTT, 50 mM NaCl, 50 μ g/ml BSA).

10 The enzymatic assay was performed essentially as described (Streuli, M. et al., 1989 Proc. Natl. Acad. Sci. USA 86:8698-8702). The immune complexes were resuspended in 50 μ l M17.6 buffer (containing 1 mM vanadate where indicated) to which had been added 15 10 μ l [32 P]tyrosine phosphorylated myelin basic protein (approximately 12 μ M). The [32 P]-tyrosine-phosphorylated myelin basic protein had been produced by in vitro phosphorylation using EGF-receptor immunoprecipitated from A431 cells. The reactions 20 were incubated for 15 minutes at 37°C with shaking, stopped with 750 μ l of an acidic stop mix containing activated charcoal, and the amount of released free [32 P]-phosphate was measured.

25 7.1.5. ENDOGLYCOSIDASE F TREATMENT

Cultures of cells transfected with RPTP κ cDNA were lysed in 1% SDS at 100°C for 3 minutes. The total cell lysates were sonicated 3 times at full 30 speed, then diluted with distilled water to decrease the concentration of SDS to 0.1%. The cell lysates were incubated at 37°C for 18 hours in the presence of 0.2 units endoglycosidase F (Boehringer- Mannheim), 0.25 M sodium acetate, pH 5.2, 20 mM EDTA, 10 mM β -35 mercaptoethanol and 0.6% NP-40. The total enzyme-treated lysate was directly loaded onto SDS-PAGE gels,

which were run, transferred to nitrocellulose and blotted with antiserum 116 or antiserum 122 as indicated.

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7.1.6. SITE-DIRECTED MUTAGENESIS

In vitro site-directed mutagenesis was performed using a commercially available kit from Clontech, 10 using the manufacturer's instructions. An oligonucleotide having the sequence CTACACCCACATCTAACGAACCGTGAAGCAGGG (SEQ ID NO:7) was used to modify the amino acid sequence RTKR in the cleavage site to the sequence LTNR. Mutagenesis was 15 confirmed by direct DNA sequencing.

15

7.1.7. IN SITU HYBRIDIZATION OF RPTPK cDNA TO RAT TISSUES

20 Sprague-Dawley rats were sacrificed by decapitation, and their brains were removed and sectioned into 20 μ m sections in a cryostat. Sections were postfix fixed in 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4, for 20 min.

25 A 50 base oligonucleotide complementary to residues 1493-1543 of the isolated RPTPK cDNA sequence (SEQ ID NO:3) was used as a probe. The oligonucleotide was labeled with $[(\alpha-^{35}S)]dATP$ (NEN, DuPont) using terminal deoxynucleotidyl transferase (Boehringer Mannheim) and purified using Sephadex G25 30 quick spin columns (Boehringer Mannheim). The specific activity of the labeled probe was from 2×10^8 to 5×10^8 cpm/ μ g DNA. Prehybridization and hybridization were carried out in a buffer containing 50% deionized formamide, 4X SCC, 1X Denhardt's 35 solution, 500 μ g/ml denatured salmon sperm DNA, 250 μ g/ml yeast tRNA and 10% dextran sulfate.

The tissue sections were incubated in a humidified environment for 14-18 h at 42-46°C in hybridization solution containing the labeled probe and 10 mM dithiothreitol. Specificity controls were 5 performed on adjacent sections by adding to the labeled oligonucleotide a 100-fold excess of the unlabeled oligonucleotide. After incubation, sections were washed in 2 changes of 2X SSC at room temperature 10 for 1 h, then in 1X SCC at 50°C for 30 min, 0.5X SCC at 50°C for 30 min, and in 0.5X SCC at room temperature for 10 min. Sections were dehydrated and exposed to X-Omat film for 3 weeks.

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7.2. RESULTS AND DISCUSSION

7.2.1. EXPRESSION OF RPTPK IN ADULT TISSUES

Northern blot analysis on adult mouse tissues 20 (FIG. 6) revealed that RPTPK expression is broad. Two major transcripts of 5.3 and 7.0 kb were detectable at different levels in all examined tissues except in spleen and testis. Particularly high levels of the 5.3 kb transcript were seen in liver and kidney 25 tissue. An identical pattern was detected using as a probe both an N-terminal and central part of the cDNA. Although the 5.3 kb size is similar to the 5.7 kb described for mRPTP (Gebbink et al., *supra*), RPTPK appears to be much more widely expressed than mRPTP μ . 30 Expression of the latter is virtually restricted to lung and, at lower levels, brain and heart.

7.2.2. TRANSIENT EXPRESSION AND ENZYMATIC ACTIVITY OF RPTPK

35 As described above, the RPTPK coding sequence was cloned into an expression vector under the control of

the CMV enhancer and promoter after manipulation to remove the untranslated leader sequence. The construct was transiently transfected into HeLa cells which were metabolically labeled with [³⁵S]-methionine, 5 lysed and subjected to a radioimmunoprecipitation assay. The antibody probe was an antiserum raised against a peptide located in the N-terminus of the protein (residues 60 to 76). This antiserum precipitated a protein of about 210 kDa from RPTPK transfected cells, but not from mock transfected cells (transfected with an "empty" expression vector) (FIG. 10 7). This immunoprecipitation was blocked by inclusion of the antigenic peptide in the immunoprecipitation reaction (lanes 3 and 6), but not by inclusion of a 15 heterologous peptide corresponding to the first catalytic homology domain of RPTPK.

To confirm that the protein encoded by the RPTPK cDNA had PTPase enzymatic activity, immune complexes 20 from transfected cells were incubated in an appropriate buffer with [³²P]-tyrosine phosphorylated myelin basic protein as a substrate. As shown in FIG. 8, approximately 3-fold higher PTPase activity was 25 detectable in immune complexes from RPTPK transfected cells as compared to control cells. This PTPase activity could be significantly inhibited by vanadate.

7.2.3. IN SITU HYBRIDIZATION ANALYSIS
OF RPTPK EXPRESSION IN THE
DEVELOPING AND ADULT CENTRAL
NERVOUS SYSTEM

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The level of expression of RPTPK mRNA was generally higher in the developing than in the adult central nervous system (CNS). At embryonic day 18 35 (E18) and at E20, the RPTPK mRNA levels were highest in the cerebral cortex and hippocampal formation,

followed by the cerebellum, brain stem and spinal cord. In the rest of the embryo, the highest levels were found in the liver, kidney and intestine (left 5 panel, FIG. 13). At postnatal day 6 (P6) and P8, expression was maximal in the cortex, olfactory bulb and hippocampal formation, especially in the dentate gyrus and CA3. In the cerebellum, the expression was highest in the granular cell layer, which in this 10 stage of development still occupies the outermost cell layer of the cerebellum (right panel, FIG. 13).

In the adult rat, expression was lower, but was clearly visible in the olfactory bulb and throughout the cortex, including the pyriform and cingulate 15 cortex. Expression of the RPTP κ mRNA was also observed in the hippocampal formation. Interestingly, expression in the cerebellum was barely detectable in the adult. This was in marked contrast with the distinct pattern and high level of expression observed 20 at P6 and P8, a period of active cerebellar development.

The *in situ* hybridization studies confirmed the expression of the RPTP κ in several organs. In addition, they demonstrated that, in the CNS, RPTP κ is 25 expressed in specific areas in a developmentally regulated manner. The levels of RPTP κ expression are higher in the actively developing areas, but expression persists in the adult, mainly in certain areas of the cortex and in the hippocampal formation. 30 These findings are consistent with the idea that CNS RPTPases are actively involved in development and plasticity. Studies on the expression of RPTPs in *Drosophila* have led to similar suggestions (Tian et al., *supra*; Yang et al., *supra*).

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8. EXAMPLE: CHROMOSOMAL LOCALIZATION OF
THE MURINE RPTPK GENE

The method was essentially as described previously (Sap, J. et al., 1990 Proc. Natl. Acad. Sci. USA 87:6112-6116; Silver, J., 1985 J. Hered. 76:436-440; Taylor, B., 1978, In: H.C. Morse, III (ed.), *ORIGINS OF INBRED MICE*, Academic Press, New York, pp. 423-438; Taylor, B.A., 1989 In: M.F. Lyon et al., eds, *GENETIC VARIANTS AND STRAINS OF THE LABORATORY MOUSE*. Oxford University Press, New York, pp. 773-796). Southern blotting analysis of *Taq* I-digested mouse genomic DNA with the 604 RPTPk probe revealed an array of 12 fragments that appeared invariant between the inbred strains surveyed, as well as a smaller set of variable bands that were used to define two allelic forms of the gene:

(1) a was defined by the presence of 1.9, 3.5 and 3.8 kb fragments and was present in inbred mouse strains AKR/J, C3H/HeJ, DBA/J, SM/J; and

(2) b was defined by the presence of a 4.1 kb fragment and was present in inbred mouse strains C57BL/6J, O20/A, C57L/J, SWR/J, SJL/J, BALB/cJ, STS/A, NZB/B1NJ).

Analysis of the inheritance pattern of this variant among recombinant inbred strains of mice (Table I), and comparison of strain distribution patterns thus obtained with those generated previously for other genetic markers, revealed close linkage between RPTPk and two markers of proximal chromosome 10: *D10Mit3* (2 discordancies among 22 strains typed, indicating a distance of 2.6 cM between the loci (0.3 cM to 13.0 cM defined 95% confidence limits); and *Ly-41* (0 discordancies among 30 strains typed, indicating a distance between the loci of < 3.5 cM at 95% confidence). The gene symbol *Ptpk* is proposed by the

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inventors, consistent with the symbol Ptpa previously assigned to RPTP α (Sap et al., *supra*).

This region of mouse chromosome 10 contains 5 multiple genes with human homologues mapping to chromosome 6q. Based on synteny, this would predict a localization of the human RPTP κ homologue to 6q, in contrast to 18pter-q11 for human RPTP μ (Gebbink et al., *supra*).

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TABLE I**DNA FRAGMENT LENGTH VARIANT ASSOCIATED WITH THE MOUSE RPTPK GENE.**

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A. Alleles and strain distribution patterns

	<u>Allele (Size kb)</u>	<u>Strains</u>
	a 1.9 + 3.5 + 3.8	AKR/J, C3H/HeJ, DBA/2J, SM/J AKXL-5,6,7,8,17,21,25,28,29, 37,38
10		BXD-1,2,5,14,15,18,21,23,25, 28,32
		BXH-2,4,7,8,12,14,19
		NXSM-D,L,W,X
		OXA-4,5,7,8,13
15	b 4. 1	C57BL/6J, O20/A, C57L/J, SWR/J, SJL/J, BALB/CJ, STS/A, NZB/B1NJ AKXL-9,12,13,14,16,19,24 BXD-6,8,9,11,12,13,16,19,20, 22,24,27,29,30,31 BXH-3,6,9, 10, 11 NXSM-C,E,F,I,N,P,Q,T1,T2,U,Z OXA- 1,2,3,6,9,10,11,12,14
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B. Linkage of ptpk to other markers typed in Recombinant Inbred strains

	<u>Marker</u>	<u>Chr</u>	<u>R/N</u>	<u>Odds</u>	<u>Distance (cM)</u>
25	D10Mit3	10	2/22	0.00941	2.6 (0.3-13.0)
	Ly-41	10	0/30	<0.00001	0.0 (<3.5)

A) 10 μ g quantities of liver or spleen genomic DNA were digested with λ TagI enzyme and analyzed by Southern blotting with the 604 RPTPK probe as described previously to define two alleles of the ptpk gene and to follow their inheritance in panels of recombinant inbred (RI) strains of mice.

B) The resulting strain distributions were compared with those previously determined for other loci in these panels of mice. Two matches were found that were unlikely to be due to chance at a 5% confidence level. For each of these, the number of non-matching RI strains found (R) is shown as a fraction of the total number of RI strains typed (N) for the two markers, together with the odds of observing that number of non-matches or a smaller one by chance (Blank, R.D. et al., 1988 Genetics 120:1073-1083), the estimated distance between the marker and ptpk, and the 95% confidence limits for that estimate (Silver, *supra*; Taylor, 1978, *supra*).

9. EXAMPLE: POSTTRANSLATIONAL PROTEOLYTIC PROCESSING OF RPTPK

5 During experiments designed to achieve stable expression of RPTPK in 3T3 cells, the present inventors observed the generation of a product of an unexpected, smaller size as well as the generation of aberrantly-sized products upon transient transfection
10 of COS cells.

15 The present inventors noted the presence of a proteolytic cleavage signal in the extracellular domain of RPTPK, (RTKR, residues 640 to 643, in the fourth FN-III repeat; FIG. 3) and wished to examine its significance in light of these observations. Thus, additional experiments were performed in COS cells transfected by the DEAE-dextran technique.

20 In order to detect cleavage products which may have accumulated, total cell lysates were directly loaded onto SDS-PAGE gels, run in electrophoresis, transferred to nitrocellulose, and immunoblotted with the two different anti-RPTPK peptide antisera (described above) specific for either the N-terminus
25 or for an epitope near the first PTPase homology domain in the intracellular portion.

30 In lysates from transfected cells, but not from mock transfected cells, both antisera recognized the same 210 kDa protein described above. The antiserum specific for the N terminus also recognized a smaller 110 kDa protein. The antiserum specific for the cytoplasmic region recognized a smaller 100 kDa protein (FIG. 9).

35 The three polypeptides (210, 110 and 100 kDa) were further characterized by subjecting the total cell lysates to endoglycosidase F digestion before

SDS-PAGE and immunoblotting. Such a treatment would be expected mainly to affect the mobility of a protein containing the glycosylated extracellular domain.

5 Following endoglycosidase F treatment, the mobility of the 210 kDa and 110 kDa proteins was significantly reduced, to 160 kDa and 89 kDa respectively. In contrast, the mobility of the 100 kDa band detected with antiserum 122 specific for an epitope in the 10 intracellular domain) was only slightly affected, suggesting that the 100 kDa peptide includes a minor glycosylation component (FIG. 9).

15 The above results, as well as pulse-chase analysis shown in FIG. 10, are consistent with the cleavage of a 210 kDa RPTP κ precursor protein into an N-terminal 110 kDa product encompassing most of the extracellular domain, and a 100 kDa moiety containing the intracellular portion and about 100 residues of extracellular sequence (FIG. 14). A consensus site 20 for cleavage by furin, a processing endopeptidase (Hosaka et al., *supra*), is indeed located 113 amino acids upstream of the start of the transmembrane segment (RTKR, residues 640-643), which would leave 25 one potential N-glycosylation site in the C-terminal cleavage product.

30 In order to confirm directly that proteolytic cleavage occurred at the RTKR (furin-recognized) site, site-directed mutagenesis was used to mutate this site to LTNR, and the effects of this mutation on the processing of the RPTP κ precursor was examined. As shown in FIG. 12, the mutant cDNA gave rise to only a 210 kDa product. These results provide evidence that 35 the RTKR region is the likely proteolytic cleavage signal and site for processing, of the RPTP κ proprotein.

The inventors next tested whether the cleavage products were associated. This was accomplished by performing an immunoprecipitation with antiserum 116, 5 specific for the extracellular 110 kDa product, on lysates of cells transfected with the wild type (wt) RPTP κ cDNA. Immunoblotting of this precipitate with antiserum 122, specific for an intracellular RPTP κ epitope, detected the presence of the 100 kDa C- 10 terminal cleavage product in the precipitate (FIG. 12). This observation strongly suggested that at least a portion of the two RPTP κ cleavage products remained associated after cleavage, and that the 100 and 110 kDa species may be considered as subunits of a 15 single complex (FIG. 14). Experiments with a secreted form of the extracellular domain of RPTP κ suggested that this association was not mediated by a disulfide linkage, since no association could be detected using SDS-PAGE under nonreducing conditions.

20 A similar posttranslational processing event has been described for the RPTPase LAR and for the Ng-CAM protein (Burgoon, M. et al. 1992. J. Cell Biol. 112:1017-1029; Streuli, M. et al., 1992 EMBO J. 11:897-907; Yu, Q. et al., 1992 Oncogene 7:1051- 25 1057). In addition, a potential cleavage site exists in the corresponding position in mRPTP μ (Gebbink et al., *supra*). It is therefore likely that proteolytic processing of RPTPs may be a more general phenomenon.

30 Such cleavage, as described above, may allow controlled shedding of the N-terminal 110 kDa subunit, and thus render the membrane-bound 100 kDa form of RPTP κ insensitive to modulation by binding of proteins in the cellular environment. Alternatively, shedding 35 might release a soluble species which retains binding activity to the putative RPTP κ ligands.

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Interestingly, secreted soluble forms of extracellular domains have been described for tyrosine kinase receptors such as the FGF-receptor (Johnson, D.E. et al., *Molec. Cell. Biol.* 11:4627-4634 (1991)). However these secreted forms were generated by an alternative splicing mechanism.

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10. EXAMPLE: ISOLATION AND ANALYSIS OF
HUMAN RPTPK (MCP7) cDNA CLONES

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10.1. PCR AND cDNA CLONING METHODS

Poly(A)+ RNA was isolated from SK-BR-3 cells (ATCC HTB30) and cDNA synthesized using avian myeloblastosis virus (AMV) reverse transcriptase as described (Sambrook, J. et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989)). Polymerase chain reaction using a pool of degenerated oligonucleotides based on two highly conserved regions of the PTPase domain (Vogel, W. et al., *Science* 259:1611-1614 (1993) was performed under standard conditions, and PCR products were subcloned in Bluescript KS+ vector (Stratagene). Sequence analysis was done by the dideoxynucleotide chain termination method (Sanger et al., 1977) using Sequenase (United States Biochemical). A lambda ZAP 11 LIBRARY (Stratagene) from SK-BR-3 poly(A)+ RNA was screened with a PCR fragment probe under high stringency conditions (Ullrich, A. et al., 1985, *Nature* 313:756-711).

10.2. RESULTS

5 The complexity of PTPases expressed in the human
breast cancer cell line SK-BR-3 was examined by
performing a PCR analysis. The primers were
degenerate sequences corresponding to conserved
sequences within the PTP catalytic domains shared by
all identified PTPases (Vogel, W. *et al.* 1993, *Science*
10 259:1611-1614). Sequence analysis of the cloned PCR
products revealed the presence of several known
PTPases, including PTP1B, LAR, TC-PTP, PTP δ , PTP ϵ ,
PTP γ , and PTPH1, as well as some novel members of the
PTPase family.

15 One of the novel sequences, designated MCP7
("mammary carcinoma-derived PTPase, clone 7"), was
highly represented (18%) in the 121 clones examined.
The 2066 bp MCP7 PCR fragment was used to screen a λ
ZAP II SK-BR-3 cDNA library at high stringency.
20 Eleven overlapping clones spanning an overall region
of approximately 6.1 kb were analyzed, revealing an
open reading frame encoding 1444 amino acids, followed
by a 3' untranslated region of 1.8kb.

25 The nucleotide sequence of human RPTP κ (SEQ ID
NO:4) is shown in FIG. 15(1) - (3). The deduced amino
acid sequence of MCP7 (SEQ ID NO:2) is also shown in
FIG. 15(1) - (3) and displays the structural
organization of a type II transmembrane PTPase
(Fischer *et al.*, 1991, Charbonneau, H. *et al.*, *Annu.
Rev. Cell Biol.* 8:463-493 (1992)). The N-terminal
30 hydrophobic stretch of 20-26 amino acids is typical of
signal peptides (von Heijne, G., *J. Mol. Biol.* 184:99-
105 (1985)). A second region consisting of hydrophobic
35 residues is found between positions 755 and 774 and is
predicted to be a single α helical transmembrane
domain. It is followed by a short region of mainly

basic residues characteristic of a transfer stop sequence (Wickner, W.T. et al., *Science* 230:400-406 (1985)). The amino-terminal portion of the putative 5 extracellular domain contains a sequence motif, a so called MAM domain, spanning a region of about 170 residues. The MAM structural motif was recently 10 established by comparison of several functionally diverse receptors (including RPTP μ and the A5 protein) and is thought to play a role in cell adhesion (Beckmann et al., *supra*). This motif is followed by 15 one possible Ig-like domain (residues 207-277). The remaining extracellular portion contains conserved sequence motifs, indicating that it is composed of four FN-III related domains corresponding to the FN- III-like domains of LAR, PTP β and RPTP μ . The 20 extracellular domain contains 12 potential N-glycosylation sites, indicating that MCP7 is highly glycosylated. Interestingly, MCP7 contains the motif RXR/LR (residues 640-643) within the fourth FN-III domain. This motif has been described as the cleavage site for the subtilisin-like endoprotease, furin (Barr, P.J., *Cell* 66:1-3 (1991); Hosaka et al., 25 *supra*).

25 The cytoplasmic part of MCP7 is composed of two PTPase domains containing the conserved amino acid sequences typical of all known PTPases (Saito, H. et al., *Cell Growth Diff.* 2:59-65 (1991)). A particularly intriguing feature is the region linking 30 the transmembrane domain to the amino-terminal PTPase domain, which is nearly twice as large as that of most other receptor-like PTPases. A similar extended distance is shared only by the homologous PTPase, hRPTP μ (FIG. 16, lower line). The overall homology 35 between MCP7 and hRPTP μ is 77%, to which the N-

terminal and C-terminal PTPase domains contribute 91% and 86%, respectively (FIG. 16).

5 10.3. DISCUSSION

10 The extracellular domain of MCP7 is composed of one MAM domain, which is a sequence motif spanning about 170 residues, which was recently established by comparison of several functionally diverse receptors (including RPTP μ and the A5 protein) and is thought to play a role in cell adhesion (Beckmann & Bork, 1993, TIBS 18:40). The extracellular domain of MCP7 further includes one Ig-like, and four FN-type III-like segments. It therefore shares structural features with some cell adhesion molecules, permitting the classification of MCP7 into the type II PTPase class.

15 MCP7 is highly homologous to mRPTP μ which has a more restricted expression pattern in lung, brain and heart (Gebbink et al., *supra*). MCP7 is expressed as a molecule consisting of two noncovalently linked subunits, a structural feature already shown for LAR. A similar processing motif was also determined within the extracellular domain of mRPTP μ (RTKR residues 632-635), which suggest that this structural organization 20 is typical for the family of type II phosphatases. Proteolytic cleavage also occurs in the extracellular domain of the cell adhesion molecule Ng-CAM in a region containing the dibasic processing motif 25 (Burgoon, M.P. et al., *J. Cell. Biol.* 112:1017-1029 (1991)). The functional significance of this structure is not yet clear. For LAR, a shedding of the extracellular E-subunit was observed in a density-dependent manner (Streuli et al., *supra*). It is 30 likely that this shedding is due to a conformational change in the extracellular domain caused by 35

- 80 -

homophilic or hydrophilic interactions between the molecules on the surface of neighboring cells that weakens the interaction between the noncovalently 5 linked subunits. The effect of this shedding on the activity of the PTPase domains within the cells is not yet clear, but a modification of the activity of the phosphatase or a change in the sensitivity to modifying processes is probable.

10

11. EXAMPLE: TISSUE DISTRIBUTION OF
HUMAN RPTPK

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11.1. RNA EXTRACTION AND NORTHERN BLOT
ANALYSIS

Total RNA was isolated by the guanidinium isothiocyanate method (Chirgwin et al., 1979, Biochemistry 18:5294-5299) from human tissue and 20 cultured cells grown to confluence. Poly(A)+ RNA was prepared on an oligo(dT) column (Aviv & Leder, 1972, Proc. Natl. Acad. Sci. USA 69:1408-1412). 4 μ g of poly(A)+ RNA was fractionated on a 1.2% formaldehyde-agarose gel and subsequently transferred to 25 nitrocellulose filters (Schleicher & Schuell). Hybridization was performed in 50% formamide, 5x SSC, 50mM NaH₂PO₄, pH 6.8, 0.5% SDS, 0.1 mg/ml sonicated salmon sperm DNA, and 5x Denhardt solution at 42°C overnight with 1-3 x 10⁶ cpm/ml of ³²P- labeled random- 30 primed DNA (United States Biochemical). Filters were washed with 2x SSC, 0.1% SDS, and 0.2x SSC, 0.1% SDS at 45°C, and exposed 5 days using an intensifying screen at -80°C.

35

11.2. RESULTS

5 Northern blot analysis revealed a broad tissue distribution of MCP7 (FIG. 17). The 6.7 kb transcript was found at elevated levels in lung and colon tissue, and, to a lesser extent, in liver, pancreas, stomach, kidney, and placenta. No transcript was detected in spleen tissue.

10 The expression pattern of MCP7 in different mammary carcinoma-derived cell lines is shown in FIG. 18. Although MCP7 expression was observed in all of the cell lines tested, the quantity of transcripts differed significantly. A second transcript with a size of 4.9 kb was also detected in all cell lines displaying a strong signal. Moreover, MDA-MB-435 15 cells contained a specific 1.4kb mRNA that hybridized with the MCP7 probe. It is interesting to note that the intensity of the Northern hybridization signals shown in FIG. 18 correlate with the abnormal over expression of EGF-R and HER2/neu RTKs. Expression of 20 MCP7 was also detected in human melanoma cell lines and some colon-carcinoma derived cell lines.

12. EXAMPLE: TRANSIENT EXPRESSION OF HUMAN
RPTPK

25 12.1. METHODS

30 MCP7 cDNA was cloned into a cytomegalovirus early promoter-based expression plasmid (pCMV). The RTK expression plasmids used were described previously (Vogel, W. et al., 1993 *Science* 259:1611-1614). At 30 hours prior transfection, 3×10^5 cells of human 35 embryonic kidney fibroblast cell line 293 (ATCC CRL 1573), grown in Dulbecco's modified Eagle's medium (DMEM) which included 4500 mg/l glucose, 9% fetal calf serum, and 2mM glutamine, were seeded into a well of a six-well dish.

Transfections with CsCl-purified plasmid DNA were then carried out using the calcium phosphate coprecipitation technique according to the protocol of 5 Chen and Okayama (Chen, C. and Okayama, H., 1987, Mol. Cell. Biol. 7:2745-2752) with a total amount of 4 μ g, which included only 0.2 μ g expression plasmid and complemented with empty vector DNA (Gorman, C.M. et al., 1989, Virology 171:377-385; Lammers, R. et al., 10 1990, J. Biol. Chem. 265:16886-16890). At 16 hours after transfection, cells were washed once and starved with medium containing 0.5% fetal calf serum.

For metabolic labeling, MEM containing Earle's salt, but lacking L-methionine, was used instead of 15 DMEM. [35 S] methionine at 40 μ Ci/ml (1,000 Ci/mmol) was added.

Cells were stimulated with an appropriate ligand for 10 min. Epidermal growth factor (EGF) at 100 ng/ml was used to stimulate cells transfected with 20 EGF-R, HER1/2, EK-R or EP-R. Insulin at 1 μ g/ml was used to stimulate cells transfected with IR. SCF at 100 ng/ml was used to stimulate cells transfected with p145^{c-M}. After stimulation, cells were lysed in 200 μ l lysis buffer (50mM HEPES, pH7.5; 150mM NaCl, 1.5mM 25 MgCl₂, 1mM EGTA, 10% glycerol, 1% Triton X-100, 2mM phenylmethylsulfonylfluoride, 10 μ g/ml aprotinin, 1mM Na-orthovanadate). The lysates were precleared by centrifugation at 125,000 x g for 10 min at 4°C, and 1/10 of the volume of the supernatant was mixed with 30 SDS sample buffer.

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. For detection of phosphotyrosine and protein antigens on immunoblots, the ECL system (Amersham) in conjunction 35 with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibody (Biorad) was used. In

order to reprobe with other antibodies, blots were incubated for 1 hour in 67mM Tris-HCl (pH 6.8), 2% SDS, and 0.1% β -mercaptoethanol at 50°C.

5 For immunoprecipitation, radiolabelled cells were incubated with antiserum at 4°C for 2 hours, washed three times with PBS (15mM NaCl, 3 mM KCl, 80 mM Na₂HPO₄·H₂O, 1.5mM KH₂PO₄, pH 7.4) to remove unbound antibodies, lysed, and precleared by centrifugation.

10 Protein A-sepharose (Pharmacia) in a volume of 20 μ l has added and incubated for two hours on a rotating wheel at 4°C. Precipitates were washed four times with HNTG-buffer (20mM HEPES, ph. 7.5, 150 mM Na Cl, 0.1% Triton X-100, 10% glycerin), SDS-sample buffer added, and SDS-PAGE was performed. X-ray film was then exposed to the dried gel two days.

15

20 The polyclonal antiserum, Ab 116, specific for the extracellular domain of murine RPTP κ , was raised against a peptide sequence (residues 60-76) within the extracellular domain of the mouse homolog of MCP7, and which was perfectly conserved in the human sequence as described supra, in Section 10. The monoclonal antibody specific for phosphotyrosine, 5E.2, was described previously (Fendly, B.M. *et al.*, 1990, Canc.

25 Res. 50:1550-1558).

12.2. RESULTS

30 Forty eight hours after transfection of MCP7 cDNA, using a cytomegalovirus promoter-based expression vector, into 293 embryonic kidney cells, radiolabelled cells were incubated with Ab 116. Cells were washed, lysed, and the antibody-bound material 35 was immunoprecipitated.

5 MCP7 expression was found on the cell surface only, and appeared as a band having an apparent molecular weight of 185 kDa. The larger size than the calculated molecular weight of 163 kDa was probably due to extensive glycosylation of the extracellular domain.

10 Two additional bands of 97 kDa and 116 kDa were immunoprecipitated (FIG. 19, left panel, lane 1); these bands were not detectable in cells transfected with a control vector. Such lower molecular weight products were thought to be cleavage products since the extracellular domain contains a common cleavage motif (RXR/LR; residues 640-643, FIG. 15(1)-(3)). for 15 processing by the endoprotease furin. These products are similar to the cleavage products described above for murine RPTP κ . Furthermore, similar processing of the extracellular domain of LAR has been described (Streuli et al., *supra*).

20 The 116 kDa fragment, the α subunit, represents most of the extracellular domain and is highly glycosylated, as indicated by the broadness of the band upon polyacrylamide gel electrophoresis analysis and its apparent molecular weight, which exceeded the 25 calculated value, based on the sequence between residing 20 and 639, by 47kD. The 97 kDa fragment, the β subunit, corresponds to an intracellular and transmembrane domain and also includes a short extracellular segment which is thought to interact 30 with the α subunit. The relatively minor discrepancy between the observed 97 kDa molecular weight size and the calculated 91.4 kDa molecular weight of the β subunit can be explained by the presence of only one potential N-glycosylation site.

35 The α and β subunit are believed to form a stable complex, such that immunoprecipitation by an antibody

specific for the extracellular domain would detect both subunits. To confirm that the 116 kDa band corresponded to the α subunit cleavage product and not merely to a non-specifically cross-reacting species, 5 lysates from MCP7 cDNA-transfected 293 cells were subjected to Western blots using antiserum 116 specific for an N-terminal epitope. With this approach, a band of about 116 kDa as well as an unprocessed precursor were found (FIG. 19, right 10 panel, lane 1), neither of which were detected in 293 cells at comparable levels transfected with a control vector (FIG. 19, right panel, lane 2).

15 13. EXAMPLE: EXAMINATION OF PTPase
ENZYMATIC ACTIVITY OF HUMAN RPTPK

To prove that the RPTPK designated MCP7 is indeed a PTPase enzyme, the above transient expression system in 293 cells was used.

20 Coexpression of MCP7 with a panel of different RTKs representing different structural subclasses allowed the examination of more physiological substrates for the PTPase as dephosphorylation targets than those commonly used.

25 To ensure that the protein localized mainly in the membrane and to avoid an overload of the cell transport system, these transfection experiments were performed with only small amounts of plasmid compared to the original protocols (Gorman, C.M. et al.,
30 *Virology* 171:377-385 (1989); Lammers, R. et al., *J. Biol. Chem.* 265:16886-16890 (1990)). The receptors tested were mainly chimeric receptors, the respective kinase function of which was under the control of an EGF-R extracellular domain (Lee, J. et al., *EMBO J.* 35 8:167-173 (1989); Herbst, R. et al., *J. Biol. Chem.* 266:19908-19916 (1991); Seedorf, K. et al., *J. Biol.*

Chem. 266:12424-12431 (1991)). Human 293 fibroblasts were transfected with equal amounts of expression plasmids encoding for an RTK and either MCP7 or a control vector. After stimulation with the appropriate ligand for the RTK, cells were lysed, equal aliquots were resolved by SDS PAGE, and the phosphotyrosine level of the receptors was examined by immunoblotting with the anti-phosphotyrosine antibody 5E2 (Fendly, G.M. et al., Canc. Res. 50:1550-1558 (1990)).

Co-expression of I-R, EGF-R, EP-R, EK-R, and SCF-R/c-kit with MCP7 resulted in a marked decrease in the ligand-induced receptor phosphotyrosine content when compared with control transfections in which MCP7 expression plasmid had been omitted (FIG. 20, upper panel, lanes 1 and 9; lower panel, lanes 1, 5, and 9). In contrast, HER1-2 appeared to be a poor substrate of MCP7, since only weak reduction of the ligand-induced phosphorylation state of this chimera was observed (FIG. 20, upper panel, lane 5). Interestingly, the intracellularly localized, incompletely processed precursor forms of I-R, EGF-R and EP-R (FIG. 20, upper panel, lanes 2, 4 and 10, 12; lower panel, lanes 2, 4), as well as that of HER 1-2 (FIG. 20, upper panel, lanes 6, 8), were efficiently dephosphorylated, suggesting that MCP7 was present and active in the same intracellular compartments as the co-expressed RTKs before reaching the cell surface.

To verify the above effects and to rule out differences in RTK expression levels, the above blots were re-probed with RTK-specific and RPTP κ -specific antibodies. The results indicated that expression levels of the various RTKs were equivalent.

14. EXAMPLE: CORRELATION BETWEEN HUMAN
RPTPK EXPRESSION AND CELL DENSITY

5 The presence of motifs in the extracellular domain of human RPTPK that resemble motifs found in proteins involved in cell-cell and cell-extracellular matrix interactions prompted an investigation of the effect on expression level of cell density in culture.

10 An equal number of SK-BR-3 cells was distributed onto either one, two, or four 15-cm dishes and incubated for two days under standard growth conditions. When harvested after two days, cells seeded at the various starting densities were found to be 100%, 70%, and 40% confluent, respectively.

15 Poly(A)+ RNA was prepared and Northern blot analysis was conducted as described *supra*, in Section 11.1, using a probe corresponding to the extracellular domain of MCP7. The results indicated that the level of MCP7 transcripts increased with increased cell density (FIG. 21, left panel).

20 To determine whether this effect was unique to SK-BR-3 cells, an identical experiment was performed using the colon carcinoma-derived cell line HT 29. Expression of MCP7 mRNA was also found to be density-25 dependent with these cells (FIG. 19, right panel).

25 As a control, the expression of mRNA encoding the enzyme GAPDH was examined in the above cells at various densities. No density dependence of the expression of these transcripts were observed.

30 The above results support the hypothesis RPTPK, and other RPTPs of the type II and type III families, are involved in, and modulated by, cell adhesion events (Charbonneau et al., *supra*). PTPases appear to be involved in events leading to growth arrest by 35 cell-cell contact (Klarlund, *supra*). The presence of orthovanadate, a potent inhibitor of phosphatase

activity diminishes normal contact inhibition of 3T3 cells. Furthermore, PTPase activity associated with the membrane fraction of 3T3 cells increased 8 fold 5 when cells were grown to a higher density (Pallen, C.J. et al., Proc. Natl. Acad. Sci. USA 88:6996-7000 (1991)).

10 The combination of CAM motifs in the extracellular domain or RPTP κ and the intracellular PTPase activity indicates that RPTP κ may act as an important mediator of events associated with arrest of cell growth. The structural features of human RPTP κ described above, the density-dependent upregulation or 15 its expression, and its potent activity in dephosphorylating RTKs supports the emerging picture of the pivotal role of RPTP κ in growth arrest through contact inhibition, as well as a role as a tumor suppressor gene.

20 15. Example: Homophilic Binding by a Receptor Tyrosine Phosphatase

The present work investigates whether, similar to "classical" members of the CAM family, RPTPases might be capable of homophilic intercellular interaction 25 (Q. Yu, T. Lenardo, R.A. Weinberg, Oncogene 7, 1051 (1992)). Reasoning that analysis of cell adhesion by the RPTPase RPTP κ would be facilitated by its ectopic expression in a cell line likely to lack conserved ligands for a mammalian RPTPase, we stably introduced 30 an RPTP κ cDNA into *Drosophila* S2 cells. These cells have a very low capacity for spontaneous aggregation or adhesion, making them an ideal and established system for such studies (H. Kramer, R.L. Cagan, S.L. Zipursky, Nature 352, 207). Cells transfected with a 35 vector containing the RPTP κ cDNA in the sense orientation with respect to the heat-shock protein 70 (hsp 70) promoter of the vector, and induced by brief

heat treatment expressed a protein of 210 kD detectable by immunoblotting with anti-RPTP κ antiserum (FIG. 22A). This protein corresponds to the unprocessed form of RPTP κ seen in mammalian cells (Y.-P. Jiang et al. Mol. Cell. Biol. 13, 2942 (1993)). In addition, after longer expression periods, a protein species of 110 kD also appeared, suggesting that the RPTP κ protein may at least, in part, partly be processed in the Drosophila cell line in a manner similar to the way in which it is processed in mammalian cells, *i.e.*, through proteolytic cleavage by a furin type endoprotease (FIG. 22A) (Y.-P. Jiang et al. Mol. Cell. Biol. 13, 2942 (1993)). A Drosophila furin homolog has recently been described (A.J.M. Roebroek et al., EMBO J. 12, 1853 (1993)).

In order to study whether RPTP κ expression may mediate cell-cell aggregation, cells stably transfected with the RPTP κ cDNA in either the sense orientation (sense cDNA) or the antisense orientation (antisense cDNA) were tested in an aggregation assay. Uninduced and heat shock-induced cells were resuspended, subjected to rotary shaking to ensure mixing and to avoid adhesion to the vessel, and were then assayed for aggregate formation. The formation of a large number of aggregates consisting of more than 10 and up to approximately 100 cells was observed in heat-shocked sense cDNA-expressing cells only, whereas control cells (*i.e.*, antisense cDNA transfected cells or non-heat shocked cells) remained essentially single cell suspensions (FIG. 22B). Two methods of quantitation, counting of aggregates under the microscope, and determination of super-threshold particles with a Coulter-counter (FIG. 22C) confirmed this conclusion. The fact that aggregation was incomplete, with a large proportion of RPTP κ

transfected cells remaining as single cells throughout the assay period, is most likely due to the fact that the transfected cell population consisted of an uncloned pool of cells presumably differing in their 5 levels of RPTPK expression. Notably, the conditions of the assay (i.e., medium, timescale, and speed of shaking) are similar to those used to demonstrate the adhesive properties of a number of well established adhesion molecules (H. Kramer, R.L. Cagan, S.L. 10 Zipursky, *Nature* 352, 207 (1991); P.M. Snow, A.J. Bieber, C. Goodman, *Cell* 59, 313 (1989)). Therefore, in view of the difficulty of measuring binding 15 affinities of many cell adhesion molecules which rely on cooperativity, it is likely that the strength of cell-cell-interaction conferred by expression of RPTPK is comparable to that of established, "classical", cell adhesion molecules.

The above experiments were performed with a full-length RPTPK cDNA, leaving unclear whether the 20 phosphatase activity of the intracellular domain is required to confer adhesive properties. In several instances, an intact intracellular domain of cell adhesion molecules has in fact been shown to be required for certain aspects of cell-cell interaction 25 (A. Nafaguchi and M. Takeichi, *EMBO J.* 7, 3679 (1988); S.H. Jaffe et al., *Proc. Natl. Acad. Sci. USA* 87, 3589 (1990), R.O. Hynes, *Cell* 69, 111 (1992)). To test this issue, a cDNA encoding a mutant protein lacking most of the intracellular, catalytic, domain of RPTPK was 30 constructed. Fig. 22D shows that such a truncation did not negatively interfere with cell aggregation as measured in this type of assay. The role of the furin cleavage site in the extracellular domain of RPTPK was also tested. Mutation of this site also left the 35 adhesive behavior intact, suggesting that cleavage of

the RPTPK proprotein (Y.-P. Jiang et al. Mol. Cell. Biol. 13, 2942 (1993)) is not required for induction of cell aggregation.

Cell adhesion molecules have been described which 5 either do (e.g. cadherin family members and integrins), or do not (e.g. N-CAM, Ng-CAM) require the presence of Ca^{++} (G.M. Edelman, Immun. Rev. 100, 11 (1987); A.F. Williams and A.N. Barclay, Annu. Rev. Immunol. 6, 381 (1988); M. Grumet, Curr. Opin. Neurobiol. 1, 370 (1991), R.O. Hynes, Cell 69, 111 (1992), B. Geiger and O. Ayalon, Annu. Rev. Cell Biol. 8 (1992)). The experiments presented in FIG. 22 were performed in the presence of 10 mM Ca^{++} in the aggregating cell suspension. Performing a similar 10 experiment in the absence of calcium ions and in the presence of 1 mM EGTA revealed no calcium requirement 15 for RPTPK mediated cellular aggregation under the conditions of the assay.

The observed aggregation correlating with 20 expression of RPTPK could be accounted for by either a homophilic binding mechanism, in which cell-cell binding is mediated by interaction between RPTPK proteins on different cells within aggregates, or by binding of the RPTPK protein to a second cell-surface 25 ligand intrinsic to the parental transfected cells. It was possible to distinguish between these two hypotheses by marking different populations of cells with the fluorescent lipophilic dye 1,1'-dioctadecyl-3,3',3'tetramethylindocarbocyanine perchlorate (diI) 30 (J. Schlessinger et al. Science 195, 307 (1977)), and then testing them for their ability to co-aggregate. In these experiments, RPTPK expressing and non-expressing cells were labeled with diI, mixed with unlabeled cells of either RPTPK expressing or non-expressing types, and the presence of cells of either 35

type in the aggregates formed was monitored by fluorescence microscopy. The results are illustrated in FIG. 23. Strikingly, mixing of unlabeled, RPTP κ positive cells with labeled, RPTP κ negative cells led 5 to the formation of aggregates consisting exclusively of unlabeled cells. Conversely, when the RPTP κ expressing cells were labeled and allowed to aggregate with unlabeled control cells, aggregates consisted entirely of labeled cells, demonstrating that dII 10 labeling does not interfere with the aggregation capacity of the transfected cells. Mixing of labeled and unlabeled cells, both expressing RPTP κ , led to the formation of mixed aggregates consisting of cells of either staining type, thus confirming that both dII 15 stained and unstained cells have the ability to co-aggregate. These results suggest that aggregation of the RPTP κ transfected cells requires the presence of the protein on all cells within the aggregate, implying a homophilic binding mechanism.

20 It was next determined whether the extracellular domain of RPTP κ was able to function by itself as a substrate for attachment of cells expressing the RPTP κ protein independent of other factors to assist in the adhesion process. A baculovirus expression 25 system was used to produce a soluble recombinant protein consisting of virtually the entire extracellular domain of the RPTP κ protein, fused to placental alkaline phosphatase, which served as a tag for purification and detection (J.G. Flanahan and P. 30 Leder, Cell 63, 185 (1990)). Fusion between the two protein moieties was designed to occur precisely before the furin proteolytic cleavage signal in the fourth fibronectin type III repeat in RPTP κ (Y.-P. Jiang et al. Mol. Cell. Biol. 13, 2942 (1993)). The 35 purified recombinant protein (K2AP) was used to coat

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bacteriological Petri dishes, and monitored for its ability to allow attachment of RPTP κ -expressing S2 cells. Only induced, RPTP κ expressing cells showed adhesive behavior to the K2AP coated surface (FIG. 24; 5 Table II below).

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TABLE II

Cell type:	S2 control un-induced	S2 control induced	S2-R-PTP- κ un-induced	S2-R-PTP- κ induced	L6	L6R- PTP- κ
5						
	Protein					
	K2AP-a	-	-	+++	+	++
	K2AP-b	-	-	+++	+	++
10	AP	-	-	-	-	-
	HER	-	-	-	-	-
	BSA	-	-	-	-	-
15	Fibro-nectin	+++	+++	+++	+	+
	poly-lysine	n.d.	n.d.	n.d.	+++	+++

Summary of adhesion data of different cell types to surfaces coated with purified K2AP protein, or other proteins (-:no cells attached; + : 50-150 cells; ++ 150-500; +++:>500; n.d. : not determined)

20 K2APa: K2AP protein purified by elution from affinity column at alkaline pH.

K2APb: K2AP protein purified by elution from affinity column using 50 % ethylene glycol.

25 AP: alkaline phosphatase control protein (J.G.

Flanahan and P. Leder, Cell 63, 185 (1990)),
25 corresponding to the tag portion of the K2AP fusion protein.

30 HER: Human EGF-receptor extracellular domain affinity-purified from a baculovirus expression system (I. Lax et al., J. Biol. Chem. 266, 13828 (1991)).

BSA: bovine serum albumin.

L6-R-PTP κ : a clone of L6 cells stably transfected with the R-PTP κ protein.

No attachment occurred to control coated surfaces, which included alkaline phosphatase or the recombinant extracellular domain of human EGF-receptor 5 (I. Lax et al., J. Biol. Chem. 266, 13828 (1991)), also purified by affinity chromatography from a baculovirus expression system. Whereas the above experiments were performed in the context of insect 10 cells, the effect of RPTP κ protein expression in mammalian cells in a similar cell-to-substrate adhesion assay was also tested. In contrast to parental Drosophila S2 cells, rat L6 myoblast cells, the mammalian cell line used as a recipient for RPTP κ overexpression, already shows a low level of 15 spontaneous adhesion to a K2AP protein coated surface. However, stable overexpression of an RPTP κ cDNA in these cells led to a significant (2.7 fold +/-1.0; n=3) increase in adhesive capacity to a surface coated with the recombinant soluble extracellular domain of 20 the RPTP κ protein (FIG. 24).

15.1 Discussion

Cell-cell contact is generally considered to play a critical role in various aspects of malignancy. For 25 example, escape from contact inhibition is a classical parameter of transformation, and, additionally, many links between cell-cell interactions and such phenomena as tumor invasion and metastasis are apparent (F. Van Roy and M. Mareel, TICB 2, 163 30 (1992)). The above data clearly demonstrate that an RPTPase of the LAR-like subfamily (containing a combination of Ig and fibronectin type III domains) is capable of homophilic binding between neighboring cells, leading to the identification of a function for 35 the extracellular domains of such molecules. This

makes it likely that other members of this RPTPase subfamily can behave in a similar fashion, and extends the series of links that have recently emerged between the adhesive properties of cells, and signal

5 transduction pathways involving tyrosine phosphorylation. For instance, adherens junctions correspond to sites of increased tyrosine phosphorylation and appear to be subject to its control, and reagents directed at integrins or

10 extracellular domains of established CAMs have been shown to elicit changes in cellular tyrosine phosphorylation (J.R. Atashi et al., *Neuron* 8, 831 (1992); T. Volberg et al., *EMBO J.* 11, 1733 (1992); R.L. Juliano and S. Haskill, *J. Cell Biol.* 120, 577 (1993)). In addition, reagents directed toward cell adhesion molecules are known to activate a number of second messenger signals (Schuch, U. Lohse, M. Schachner, *Neuron* 3, 13-20 (1989); P. Doherty, S.V. Ashton, S.E. Moore, F. Walsh, *Cell* 67, 21 (1991)).

20 The above observation suggests mechanisms by which such signals might be generated. For example, direct cell-cell contact between RPTPases on adjacent cells could lead to local RPTPase oligomerization events affecting either the catalytic activity or

25 localization of RPTPases, which in turn have been suggested to modulate the activity of src-family tyrosine kinases (H. L. Ostergaard et al., *Proc. Natl. Acad. Sci. USA* 86, 8959 (1989); T. Mustelin and A. Altman, *Oncogene* 5, 809 (1989); X.M. Zheng, Y. Wang, C.J. Pallen, *Nature* 359, 336 (1992)). Moreover, the similar structural and functional properties of the extracellular domains of RPTPases and CAMs prompts the speculation that RPTPases may, in addition to self-interaction, also be capable of interacting

30 35 heterophilically with other molecules involved in cell

adhesion, whether in *cis* or in *trans* (G.M. Edelman, Immun. Rev. 100, 11 (1987); A.F. Williams and A.N. Barclay, Annu. Rev. Immunol. 6, 381 (1988); M. Grumet, Curr. Opin. Neurobiol. 1, 370 (1991), R.O. Hynes, Cell 5 69, 111 (1992), B. Geiger and O. Ayalon, Annu. Rev. Cell Biol. 8 (1992), M. Grumet and G.M. Edelman, J. Cell Biol. 106, 487-503 (1988); G.A. Kadmon, A. Kowitz, P. Altevogt, M. Schachner, J. Cell Biol. 110, 193 (1990); A.A. Reyes, R. Akeson, L. Brezina, G.J. Cole, Cell Reg. 1, 567 (1990); P. Sonderegger and F. G. Rathjen, J. Cell Biol. 119, 1387 (1992); M.G. Grumet, A. Flaccus, R.U. Margolis, J. Cell Biol. 120, 815 (1993)).

15 The references cited above are all incorporated by reference herein, whether specifically incorporated or not.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

20 While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions 25 following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as 30 may be applied to the essential features hereinbefore

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set forth as follows in the scope of the appended
claims.

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WHAT IS CLAIMED IS:

1. An isolated mammalian receptor-type protein tyrosine phosphatase κ protein or glycoprotein molecule.

2. The molecule of Claim 1 wherein the molecule comprises the amino acid sequence SEQ ID NO:1, shown in FIG. 3.

10 3. The molecule of Claim 1 wherein the molecule is a human receptor-type protein tyrosine phosphatase κ .

15 4. The molecule of Claim 3 wherein the molecule comprises the amino acid sequence SEQ ID NO:2, shown in FIG. 15.

20 5. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the receptor-type protein tyrosine phosphatase κ protein of Claim 1.

25 6. The nucleic acid of Claim 5 wherein the nucleotide sequence encoding the receptor-type protein tyrosine phosphatase κ protein is the nucleotide sequence SEQ ID NO:3, shown in FIG. 1(1) - 1(5).

30 7. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the human receptor-type protein tyrosine phosphatase κ protein of Claim 3.

35 8. The nucleic acid molecule of Claim 7 wherein the nucleotide sequence encoding the human receptor-type protein tyrosine phosphatase κ is the nucleotide sequence SEQ ID NO:4, shown in FIG. 15(1) - 15(5).

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9. The nucleic acid molecule of Claim 5 or 7
wherein the nucleic acid molecule is cDNA.

10. The nucleic acid molecule of Claim 5 or 7
5 wherein the nucleic acid is genomic DNA.

11. The nucleic acid molecule of Claim 5 or 7
wherein the nucleic acid molecule is an expression
vehicle.

10 12. The nucleic acid molecule of Claim 11
wherein the expression vehicle is a plasmid.

13. A prokaryotic host cell having the plasmid
15 of Claim 12.

14. A eukaryotic host cell having the plasmid of
Claim 12.

20 15. A method for preparing the receptor-type
protein tyrosine phosphatase κ protein or glycoprotein
of Claim 1, comprising:

25 (a) culturing a host cell capable of expressing
the protein or glycoprotein under culturing
conditions that permit expression
of the protein or glycoprotein; and
(b) recovering the protein or glycoprotein from
the culture.

30 16. The method of Claim 15 wherein the host cell
is a prokaryotic cell.

17. The method of Claim 15 wherein the host cell
is a eukaryotic cell.

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18. An antibody specific for the protein or glycoprotein of Claim 1.

19. The antibody of Claim 18 wherein the 5 antibody is a monoclonal antibody.

20. A method for detecting the presence of the nucleic acid molecule of Claim 5 in a cell, comprising:

10 (a) contacting the cell with a nucleic acid probe capable of specifically hybridizing to least a portion of the nucleic acid molecule of Claim 5 under hybridizing conditions; and
(b) measuring the hybridization of the probe to 15 the nucleic acid of the cell, thereby detecting the presence of the nucleic acid sequence.

21. A method for detecting the presence of the 20 nucleic acid molecule of Claim 5 in a cell, comprising:

25 (a) contacting the cell with nucleic acid primers capable of specifically binding to at least a portion of the nucleic acid molecule of Claim 5 under hybridizing conditions;
(b) selectively amplifying at least a portion of the nucleic acid molecule of Claim 5; and
(c) detecting the amplified nucleic acid of the 30 cell, thereby detecting the presence of the nucleic acid sequence.

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22. A method for detecting in a cell the presence of or measuring the quantity of a receptor protein tyrosine phosphatase κ protein or glycoprotein, comprising:

- 5 (a) contacting the cell or an extract thereof with the antibody of Claim 18; and
- (b) detecting binding of the antibody to the cell or extract thereof, or measuring the quantity of antibody bound,
- 10 thereby determining the presence of or measuring the quantity of the receptor protein tyrosine phosphatase κ protein or glycoprotein.

23. A method for identifying in a chemical or biological preparation a compound capable of binding to the receptor protein tyrosine phosphatase κ protein, glycoprotein or derivative of Claim 1, comprising:

- 20 (a) attaching the receptor protein tyrosine phosphatase κ protein, glycoprotein or derivative, or the ligand-binding portion thereof, to a solid phase matrix;
- (b) contacting the chemical or biological preparation with the solid phase matrix; allowing the compound to bind;
- 25 (c) washing away any unbound material; and
- (d) detecting the presence of the compound bound to the solid phase matrix.

30 24. A method for isolating from a complex mixture a compound capable of binding to the receptor protein tyrosine phosphatase κ protein or glycoprotein of Claim 1, comprising:

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- (a) attaching the receptor protein tyrosine phosphatase κ , or the ligand-binding portion thereof, to a solid phase matrix;
- 5 (b) contacting the complex mixture with the solid phase matrix allowing the compound to bind;
- (c) washing away any unbound material; and
- (d) eluting the bound compound,
thereby isolating the compound.

10

25. A method for identifying an agent capable of modulating the enzymatic activity of receptor protein tyrosine phosphatase κ , comprising:

15

- (a) contacting the agent with a receptor protein tyrosine phosphatase κ in pure form, in a membrane preparation, or in a whole cell;
- (b) incubating the mixture of step (a) for a sufficient interval;
- (c) measuring the enzymatic activity of the receptor protein tyrosine phosphatase κ ; and
- 20 (d) comparing the enzymatic activity to that of receptor protein tyrosine phosphatase κ incubated without the agent,

20

thereby determining whether the agent modulates
25 enzymatic activity.

30

26. The method of Claim 25 wherein the agent is capable of decreasing the activity of the receptor protein.

27. The method of Claim 25 wherein the agent is capable of inhibiting the activity of the receptor protein.

35

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28. A method for inhibiting homophilic binding of endogenous Type II receptor protein tyrosine phosphatase molecules in a mammal comprising administering to the mammal an effective amount of an 5 inhibitory compound so that the homophilic binding is inhibited.

29. The method of Claim 28 wherein the homophilic binding is intercellular.

10 30. The method of Claim 28 wherein the inhibitory compound comprises an extracellular domain of a Type II receptor protein tyrosine phosphatase molecule.

15 31. The method of Claim 28 wherein inhibiting of homophilic binding modulates the endogenous enzymatic activity of the Type II receptor protein tyrosine phosphatase molecules.

20 32. The method of Claim 31 wherein the endogenous enzymatic activity of the Type II receptor protein tyrosine phosphatase molecules is increased.

25 33. The method of Claim 31 wherein the endogenous enzymatic activity of the Type II receptor protein tyrosine phosphatase molecules is decreased.

34. The method of Claim 28 or 31 wherein 30 inhibiting homophilic binding of endogenous Type II receptor protein tyrosine phosphatase molecules regulates a cellular function comprising differentiation or cell cycle control.

35

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35. The method of Claim 28 or 31 wherein
inhibiting homophilic binding of endogenous Type II
receptor protein tyrosine phosphatase molecules
regulates cellular behavior comprising motility,
5 contact inhibition, cell adhesion, or signal
transduction.

36. The method of Claim 28 or 31 wherein
inhibiting homophilic binding of endogenous Type II
10 receptor protein tyrosine phosphatase molecules
regulates an abnormal or deleterious process
comprising cellular transformation to a cancerous
state.

15 37. A method for inhibiting homophilic binding
of endogenous receptor-type protein tyrosine
phosphatase κ molecules comprising administering to
the mammal an effective amount of an inhibitory
compound so that the homophilic binding is inhibited.

20 38. The method of Claim 37 wherein the
homophilic binding is intercellular.

25 39. The method of Claim 37 wherein the
inhibitory compound comprises a soluble receptor-type
protein tyrosine phosphatase κ protein extracellular
domain.

40. The method of Claim 37 wherein inhibiting
30 homophilic binding of endogenous receptor-type protein
tyrosine phosphatase κ protein modulates endogenous
receptor-type protein tyrosine phosphatase κ enzymatic
activity.

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41. The method of Claim 40 wherein the endogenous enzymatic activity of the receptor-type protein tyrosine phosphatase κ protein is decreased.

5 42. The method of Claim 40 wherein the endogenous enzymatic activity of the receptor-type protein tyrosine phosphatase κ protein is increased.

10 43. The method of Claim 37 or 40 wherein inhibiting homophilic binding of endogenous receptor-type protein tyrosine phosphatase κ molecules regulates a cellular function comprising differentiation or cell cycle control.

15 44. The method of Claim 37 or 40 wherein inhibiting homophilic binding of endogenous receptor-type protein tyrosine phosphatase κ molecules regulates a cellular behavior comprising motility, contact inhibition, cell adhesion, or signal 20 transduction.

25 45. The method of Claim 37 or 40 wherein inhibiting homophilic binding of endogenous receptor-type protein tyrosine phosphatase κ molecules regulates an abnormal or deleterious process comprising cellular transformation to a cancerous state.

30 46. A method for identifying a compound capable of inhibiting homophilic binding of Type II receptor-type protein tyrosine phosphatase molecules comprising:

35 (a) exposing an immobilized molecule comprising a Type II receptor-type protein tyrosine phosphatase protein extracellular domain to a Type II receptor-

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type protein tyrosine phosphatase protein molecule and a compound;

(b) removing all molecules that do not attach to the immobilized molecule; and

5 (c) determining if the Type II receptor-type protein tyrosine phosphatase protein molecule binds the immobilized molecule.

47. A method for identifying a compound capable 10 of inhibiting homophilic binding of Type II receptor-type protein tyrosine phosphatase molecules comprising exposing Type II receptor-type protein tyrosine phosphatase-expressing cells to a compound and determining if the Type II receptor-type protein 15 tyrosine phosphatase-expressing cells aggregate.

48. The method of Claim 46 or 47 wherein the Type II receptor-type protein tyrosine phosphatase molecule is receptor-type protein tyrosine phosphatase

20 x.

25

30

35

1 / 33

Signal peptide 1 atggatgtggcgccgtgcgttgctgttttagctctctggcttcgtacccgtgg 60
 1 [M D V A A A A L P A F V A L W L L Y P W 20
 61 cctctctgggtcgcccttggCcagttctcagcagggtgctgtactttgtatgtatggg 120
 21 P L L G S] A L G Q F S A [G G C T F D D G 40
 A5 homology region 121 ccaggggttgcactaccaccaggattatacgatgactttagtgggtccatgtcagt 180
 41 P G A C D Y H Q D L Y D D F E W V H V S 60
 181 ggcgcaggaaaccttattacgtggcccccggaaatgcctcaagggttccatatatgggtgtgGac 240
 61 A Q E P H Y L P P E M P Q G S Y M V V D 80
 241 tcctcaaatcatgatcctggagaaaaagccagacttcagtcgtgcctaccatgaaggagaat 300
 81 S S N H D P G E K A R L Q L P T M K E N 100
 301 gacacccactgcattgatttcagttacccgttatatagccagaagggttgaaccctggc 360
 101 D T H C I D F S Y L L Y S Q K G L N P G 120
 361 actttgaatatccttagtttagggtaataaaggaccccttgcataatccaaatttggaatgt 420
 121 T L N I L V R V N K G P L A N P I W N V 140
 421 actggattcactggcgtgattggcttcgggtgaacttagtcgtgagcaccccttggccc 480
 141 T G F T G R D W L R A E L A V S T F W P 160
 481 aatgaataccaggtaatatttgaagtcgtgaagtctcaggaggagaagtggttatattggc 540
 161 N E Y Q V I F E A E V S G G R S G Y I A 180
 541 attgatgacatccaaagtccgtgaggatccctgcgataaaatccctcatccctccgcctt 600
 181 I D D I Q V L S Y] P C D K S P H F L R L 200
 601 ggtgtgtggagggtcaatgctggcagaatGCcacatccgtgcattgtacaggaga 660
 201 G D V E V N A G Q N A T F Q C I A T G R 220
 661 gatGCtgTGcataacaaggatccgtgcagagacgcataatggagaagacataccctggcc 720
 221 D A V H N K L W L Q R R N G E D I P V A 240
 721 cagactaagaacataaaatcacagaagatttgcgtgccttcagatgtcaagaagtgaca 780
 241 Q T K N I N H R R F A A S F R L Q E V T 260
 781 aaaactgaccaggatccgttgcgtactcgtcadaacgcacgttcgtgggtttcc 840
 261 E T D Q D L Y F C V T Q S E R G S G V S 280

FIG. 1(1)

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841	aattttgcactcaactcattgtgagagaaccacccattgtcctcccaagctgcct	900
281	N F A Q L I V R E P P R P I A P P Q L L	300
901	ggtgttggccacttacttacttgcactcaactaaatgccaactctattattggcgatggc	960
301	G V G P T Y L L I Q L N A N S I J G D G	320
961	cccatcattcctgaaagaagtagagtatcgaatcaggatcttggacagaaaacccat	1020
321	P I I L K E V E Y R M T S G S W T E T H	340
1021	gcagtcaacgcaccaacatataagttgtggcatttagaccacatcagaatcggatc	1080
341	A V N A P T Y K L W H L D P D T E Y E I	360
1081	cgcgttctgttaccagacccctggcgaaggggggactgggtgcgcaggaccactgatc	1140
361	R V L L T R P G E G G T G L P G P P L I	380
1141	actagaacgaagtgtgcagaacctatgcggacacccaaagactttaaagattgtgaaatc	1200
381	T R T K C A E P M R T P K T L K I A E I	400
1201	caggcaaggcgcattgcagtggactggactggacttgcgttggctacaacatcactcggttgcac	1260
401	Q A R R I A V D W E S L G Y N I T R C H	420
1261	actttcaacgtcaactatgttgcattacttccgtggccacaatgagagcaggcagac	1320
421	T F N V T I C Y H Y F R G H N E S R A D	440
1321	tgcttggacatggacccaaagccctcagcatgtgtgaaccatctgcacccatcacaca	1380
441	C L D M D P K A P Q H V V N H L P P Y T	460
1381	aatgtcagcccaactatgttgcattacttccgtggccacaatgagagcaggcagac	1440
461	N V S L K M I L T N P E G R K E S E E T	480
1441	atcatccaaactgttgcattacttccgtggccacaatgagagcaggcagac	1500
481	I I Q T D E D V P G P V P V K S L Q G T	500
1501	tcctttgaaaacaagatcttcctgttgcattacttccgtggccacaatgagagcaggcagac	1560
501	S F E N K I F L N W K E P L E P N G I I	520
1561	atccagtatgaggtgagctatagcagcataagatcatttgcacccatgttccgtggc	1620
521	T Q Y E V S Y S S I R S F D P A V P V A	540
1621	ggccccccacagactgtatcaaatttatggaaatagtacacaccatgttccgtatctt	1680
541	G F P Q T V S N L W N S T H H V F M H L	560
1681	caccctggaaaccacccataccagttttataagagccagCactgtcaaaggcttggacca	1740
561	H I G T T Y Q F F I R A S T V E G F G T	580

FIG. 1(2)

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1741 gcaacagccatcaatgtgaccacaaatatctcagctccaagcttacctgactatgaaagc 1800
 581 A T A I N V T T N I S A P S L P D Y E G 600

1801 gttgatgcctctgtaatgaaactgccaccatcacagtactattgaggcctgcacaa 1860
 601 V D A S L N E T A T T I T V L L R P A Q 620

1861 gccaaagggtgtctatcagtgttatcaaattttgtggagcgtacacccacatcg 1920
 621 A K G A P I S A Y Q I V V E Q L H P H R 640

1921 acgaaggctgaaggcagggccatggaatgttaccaggtaaccggatccagaacgcc 1980
 641 T K R E A G A M E C Y Q V P V T Y Q N A 660

1981 ctaagtggggGcgcgCcctattactttgcgcagaacttccccctggaaatcttcccgag 2040
 661 L S G G A P Y Y F A A E L P P G N L P E 680

2041 cctgtcccttcacccgtgggtgacaacccggacataaaggctttggaaaccttccctg 2100
 681 P A P F T V G D N R T Y K G F W N P P L 700

2101 gccccccgaaaggataacaacatctatccaaaggatgtacgtggagaaggaaact 2160
 701 A P R K G Y N I Y F Q A M S S V E K E T 720

2161 aaaaacccaaatgtgtacgaaattgtacaaaaggcagcggcaacAgaagaaccagaagtgtc 2220
 721 K T Q C V R I A T K A A A T E E P E V I 740

2221 ccagacccggcaaaGCagacagacagactggtaaaaatcgccggCatcgtgtggcatc 2280
 741 P D P A K Q T D R V V K [I A G I S A G I 760

Trans-
membrane

2281 ctatgtttcatccttctctgtgtggatcatgtatgtggaaaagagcaagcttgct 2340
 761 L V F I L L L V V I V I V K K S K L A 780

2341 aagaagcgcaaaatgtcaatgggaacacacgtcaggagatgaccacatggtaatgt 2400
 781 K K R K D A M G N T R Q E M T H M V N A 800

2401 atggaccgaaggatgtgaccagagcaccctgtcatgcagaagaccccttccctcacc 2460
 801 M D R S Y A D Q S T L H A E D P L S L T 820

2461 ttcaTggaccaacataacttcagttccaagattgccaatgtccacttgcgtccactg 2520
 821 F M D Q H N F S P R L P N D P L V P T A 840

2521 gtgttagatgagaaccacacgtgccacagcagactgtgtgtggatgtccctcg 2580
 841 V L D E N H S A T A E S S R L L D V P F 860

FIG. 1(3)

2581 tacctctgcgaagggacagagtcccccttaccagacaggacagctgcacccagccatcago 2640
 861 Y L C E G T E S P Y Q T G Q L H P A I R 880

2641 gtggccgacttactgcagcacattaacctcatgaagacatcagacagctatgggttcaaa 2700
 881 V A D L L Q H I N L M K T S D S Y G F K 900

2701 gagaatacggagagCttcttgaaggccagtcagcccttggatgtggctaaaaaggat 2760
 901 E E Y E S F F E G Q S A S W D V A K K D 920

PTPase Domain I

2761 caaaacagagcaaagaaccgatacgaaacattatcgcatatgatcaactccagagtcatc 2820
 921 Q N R A K N R Y G N I I A Y D H S R V I 940

2821 ctgcaacctgttggaaagatgacccttctcagattacattaatgccaactacatcgacatt 2880
 941 L Q P V E D D P S S D Y I N A N Y I D I 960

2881 tggctgtacagggatggcttaccagagaccaagccactacattGCaactcaaggcccagtt 2940
 961 W L Y R D G Y Q R P S H Y I A T Q G P V 980

2941 catgaaaccgtatatgattttGGAgatgggtggcaagagcagtctgcctgtattgtg 3000
 981 H E T V Y D F W R M V W Q E Q S A C I V 1000

3001 atggtaactaaTttagtgGaagtTGGCCGgtgaaatgtctataatattggcctgtatgat 3060
 1001 M V T N L V E V G R V K C Y K Y W P D D 1020

3061 actgaggttatggtaacttcaaaagtcaCTGCgtagaaatggagccacttgctgagtt 3120
 1021 T E V Y G D F K V T C V E M E P L A E Y 1040

3121 gtcgttaggacattcacccggaaaggagggctataatgaaatccgtgaagtcaaacag 3180
 1041 V V R T F T L E R R G Y N E I R E V K Q 1060

3181 ttccacttcaactggctggcctgaccatgggtttccataccacgcacaggctctgtca 3240
 1061 F H F T G W P D H G V P Y H A T G L L S 1080

3241 ttatccggagagtcaagctatctaaccctcccagtgtgtggccattgtcgtaactgc 3300
 1081 F I R R V K L S N P P S A G P I V V H C 1100

3301 agtgcgtgtgtggcgcacaggctttacattgttattgacataatgtggacatggct 3360
 1101 S A G A G R T G C Y I V I D I M L D M A 1120

3361 gaaagagaggggtgtgggtgacatctacaactgtgtgaaagccttacgatctcgccgcat 3420
 1121 E R E G V V D I Y N C V K A L R S R R I 1140

3421 aatatggtacagacagggaaacactacattttattcatgatgccattttagaaggctgc 3480
 1141 N K V Q T E E Q Y I F I H D A I L E A C 1160

FIG. 1(5)

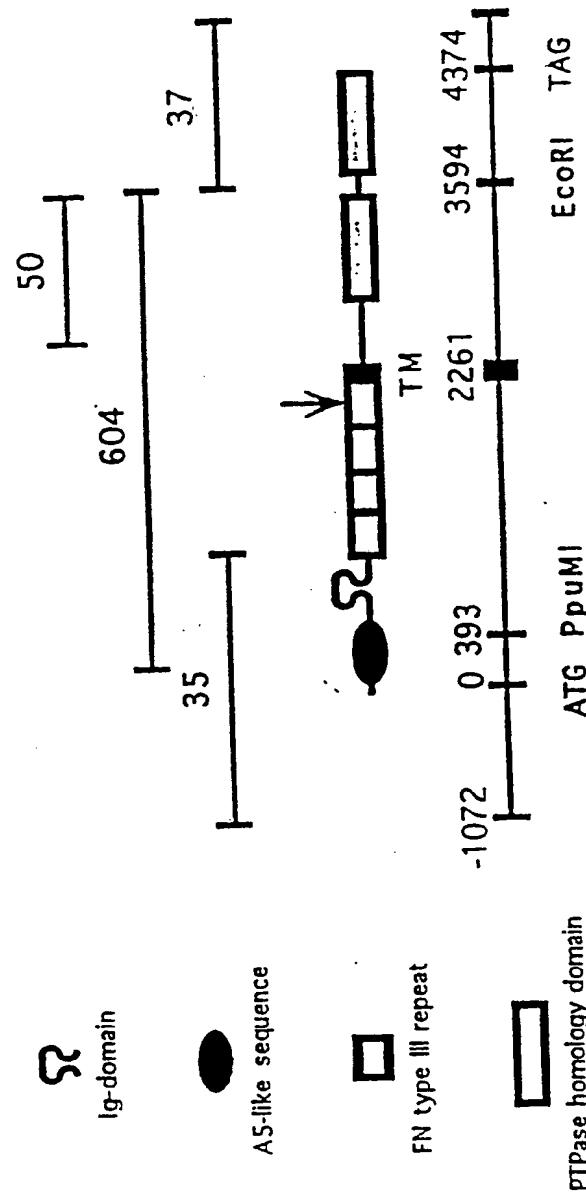


FIG. 2

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3
EIGI

I (296) P rQL L GVGPTYLILQNNNS I IGDGPILIKEVE Y RMTSGSMTEHVNAPTYKLWHLDPDTE. YEIRVLL T R PG EG G TGLRGFLITRT
II (392) P .KT L KIAEIQA..RRIAVD W ESLGYNITRCHT F NVTICHYFRGHNESRADCLDMDPKA...PQHVVNH L P PY TN V SLMKIL. THREG
III (493) P VKS L QGTSFE...NKIFLN W KEPLERNGLITQ Y EVSYSSIRSFDAVPVAGPPOTVSNLWNSTHHVFMH L H PG TT Y QFFIRASTVKGF
IV (596) P DYE G VDASLNETATTITVL L RPAQAKCAPISA Y QIVVEQLHPRTRK. EAGAMECYQV...PVTYQNA L S GO AP Y YTAMELPPGMLP
PBN-III(7) P PTN L HLEANPDT.GVLTWS W ERSTTPD..ITG Y RITTPPTNGQQNSLEEVHADQ.....SSCTFDN L S PG LE Y NWSVY..TVKOD

FIG. 4

PTP- κ (34)	GGCTFDIXPGACDYHODLYDDFEWVHVSQE.
PTP- μ (26)	PHYLPPPEMPQGSYMWVDSNNHDPEKARLQLPTMKEN.
A5 (651)	DTHCIDFSSYLLYSOKGGCLFDEPYSTCGYSQADEDFFNWEQVNLTQKPT.
Consensus	SDPWWPGSFSMLVNTSGKPEGQRAHLLLPQLKEN.
	DTHCIDFHYFVSSKCKFGWGSQKTVCNWQHDISSDLKWAVLNSKTP.
	..VQDHTGDNFIYSEADERHEGRAARLMSPVVSSSRSAHKCLTPWYHM.
	..D
	..C---D---D---W---N---T---P---G---F---E---G---F---Y

PTP- κ	GLNPGLTINILVRVN.
PTP- μ	KGPLANPIIWNVTGFTGRDWLRAELAVSTFWPNEXQVIFEAEVSGGRSGYIAIDDIQVLSY
A5	NAAPGCLLNVVKVN.
Consensus	NGPLGNPIIWN1ISGDPTRTWHRRAELAISTFWPNFYQVIFEV.
	VTSGHQQGYLAIDEVKVLGH
	GSHVGTLSIKLVEMEEDFDQTLWTVSGNQGDQWKEARVVLHKTMKQ.
	YQVIVEGTVGKGSAGGIAVDDIIIANH
	G---GTL-I---K---W-VSG---G---W---A---YQVI-E---V---G---G-IA-DDI---H

FIG. 5

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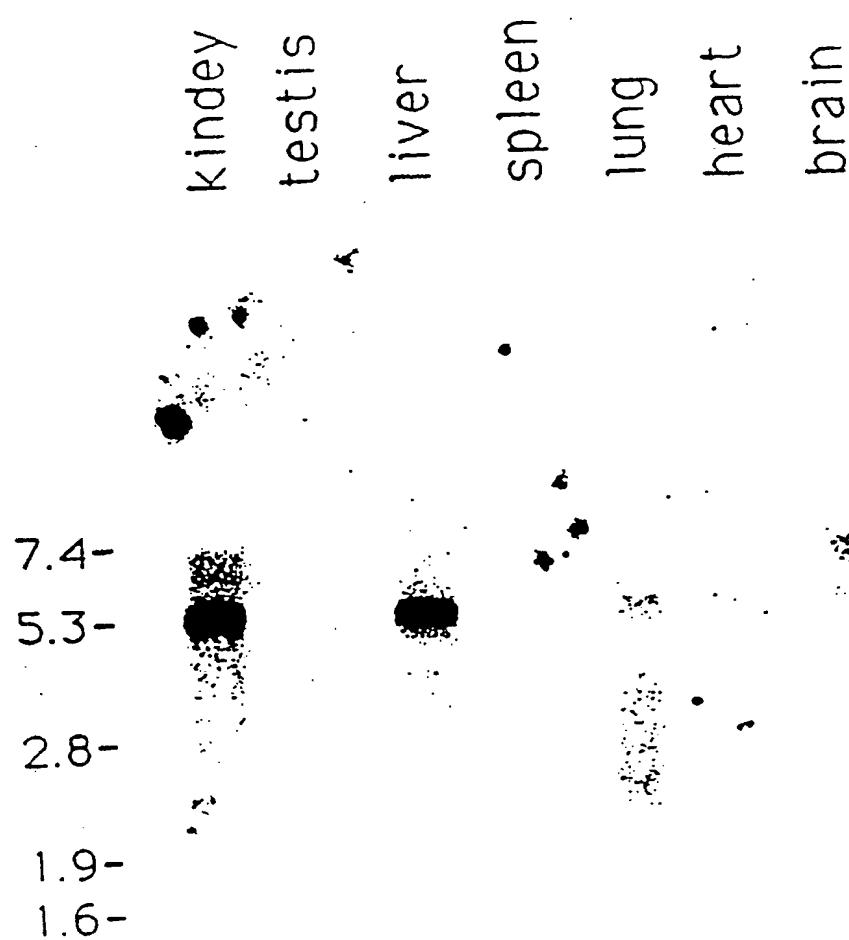


FIG. 6

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Transfected:	-	-	-	+	+	+
Antibody:	pre	α -K	α -K	pre	α -K	α -K
Peptide:	-	-	+	-	-	+

211 -



119 -



98 -



81 -



64 -

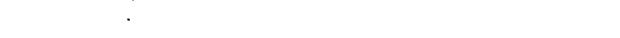
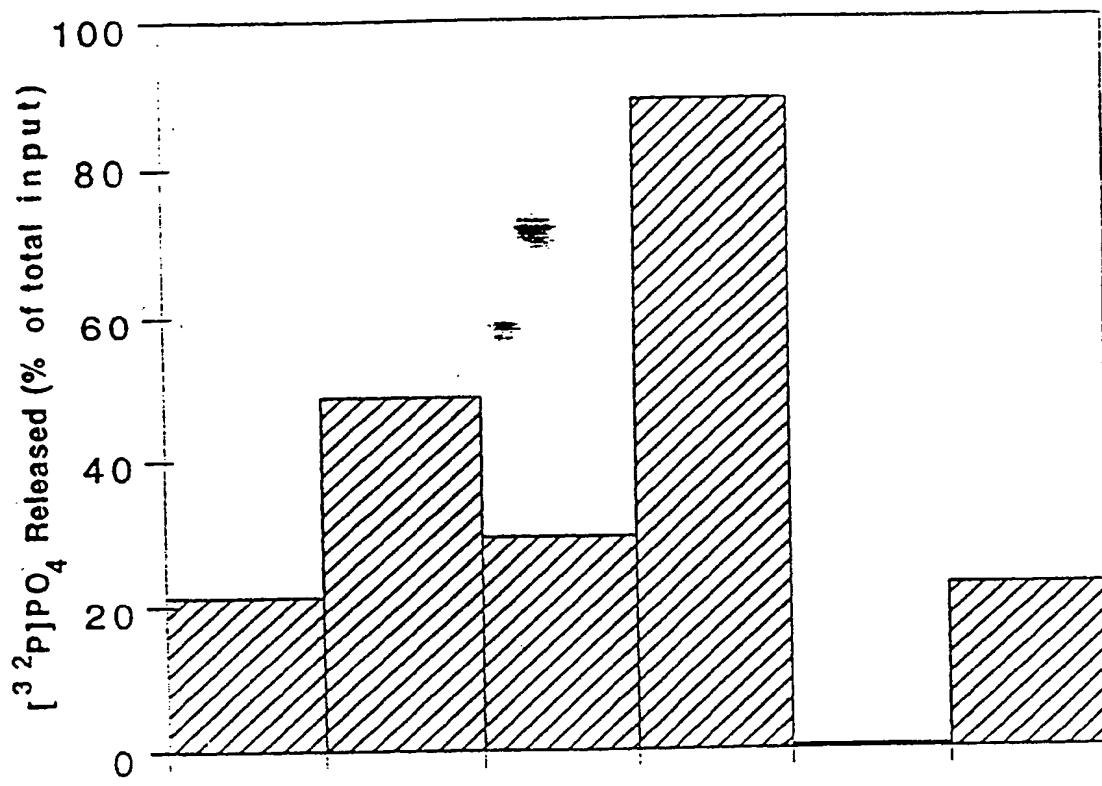


FIG. 7

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Transfection	Antibody	Vanadate
mock	pre	-
κ	pre	-
mock	116	-
κ	116	-
116	pre	-
116	116	+
116	116	+

FIG. 8

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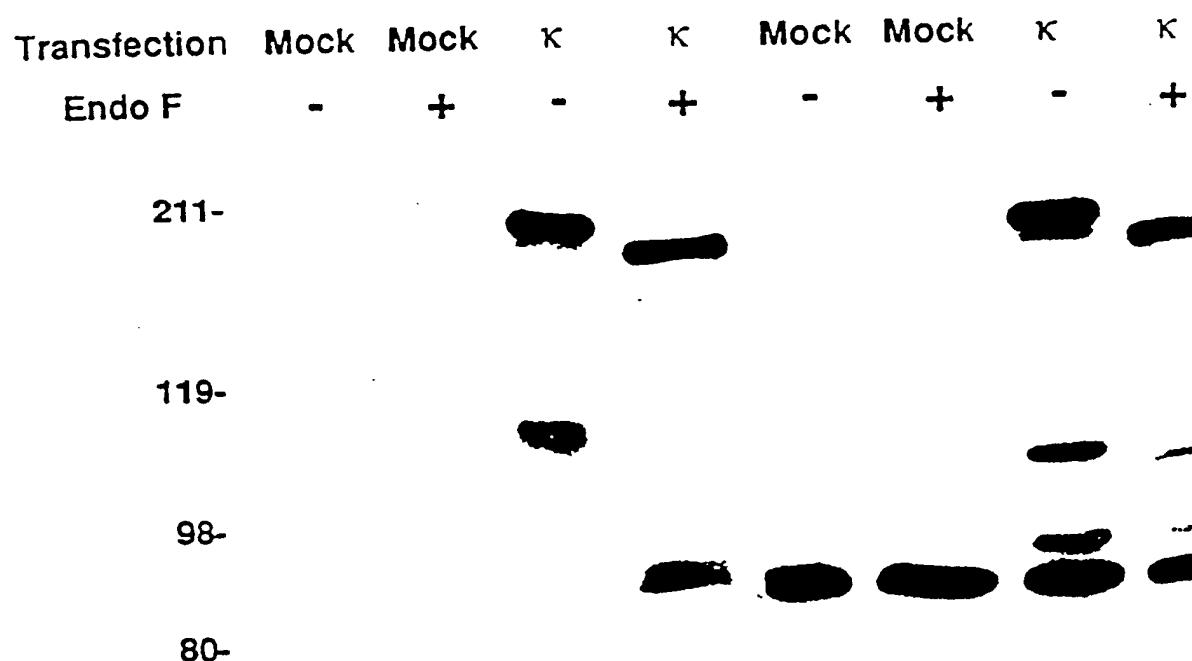


FIG. 9

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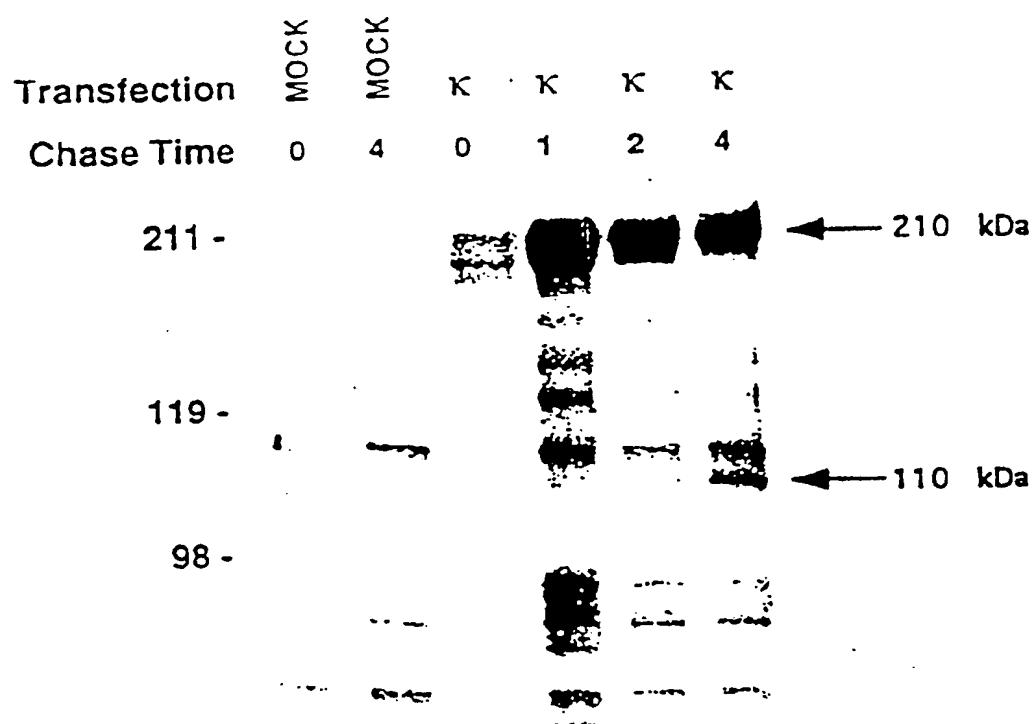


FIG. 10

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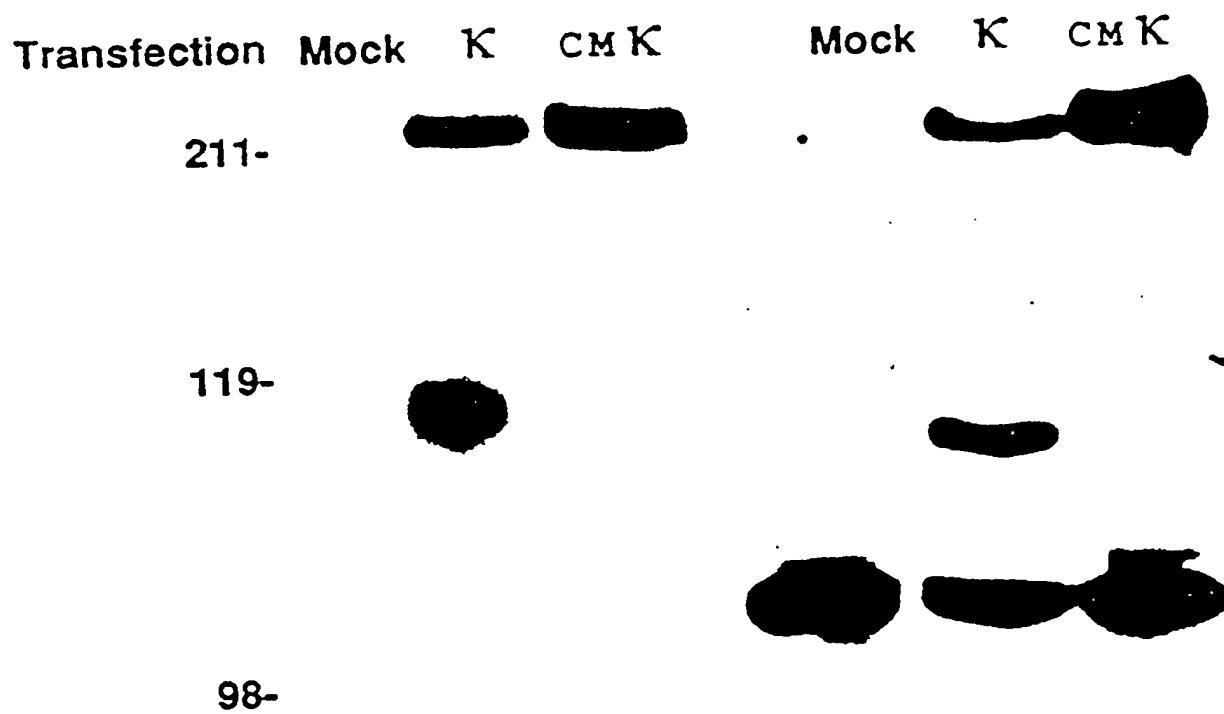


FIG. 11

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Transfection	Mock	K	K
IP	116	116	Total
			lysate

211-



119-



98-



FIG. 12

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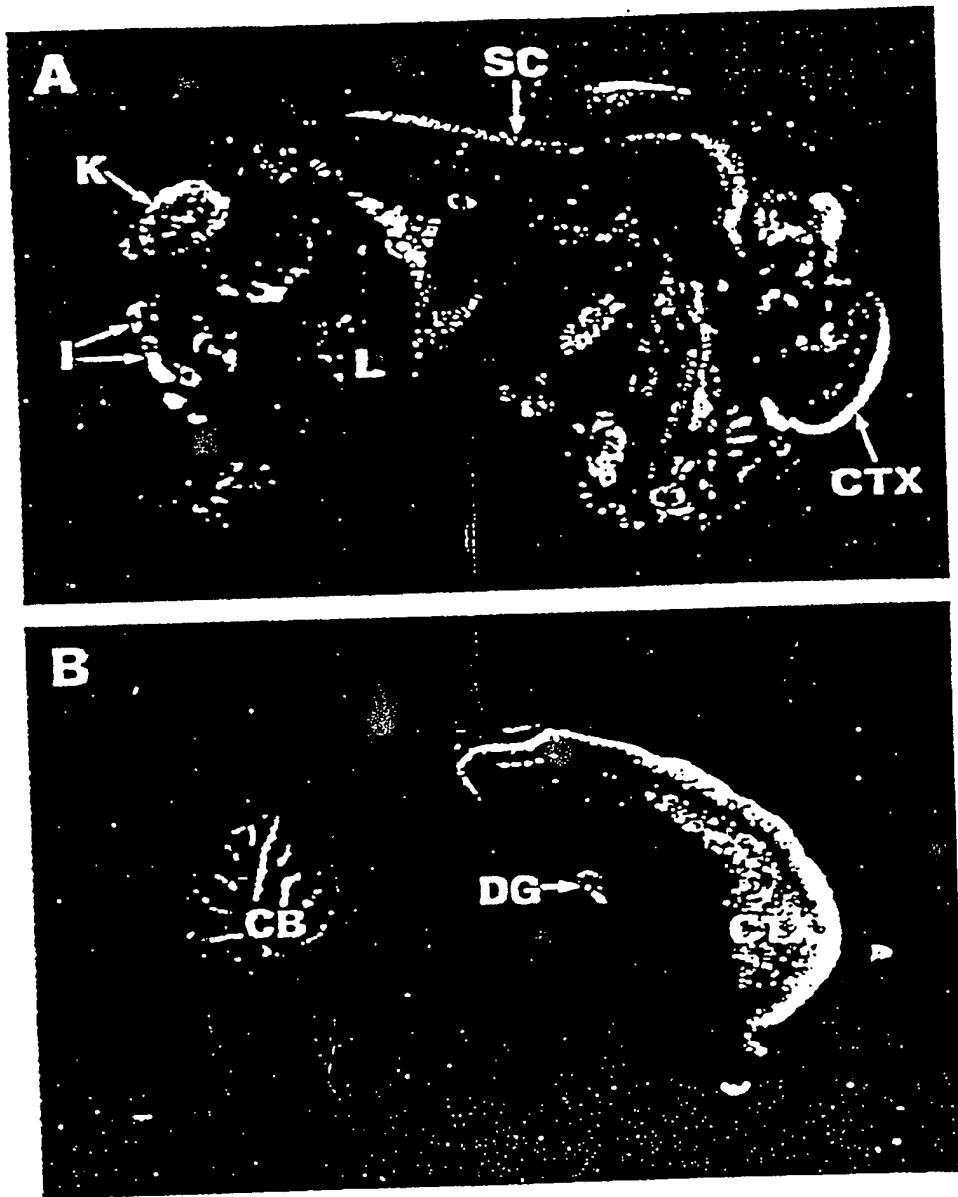


FIG. 13

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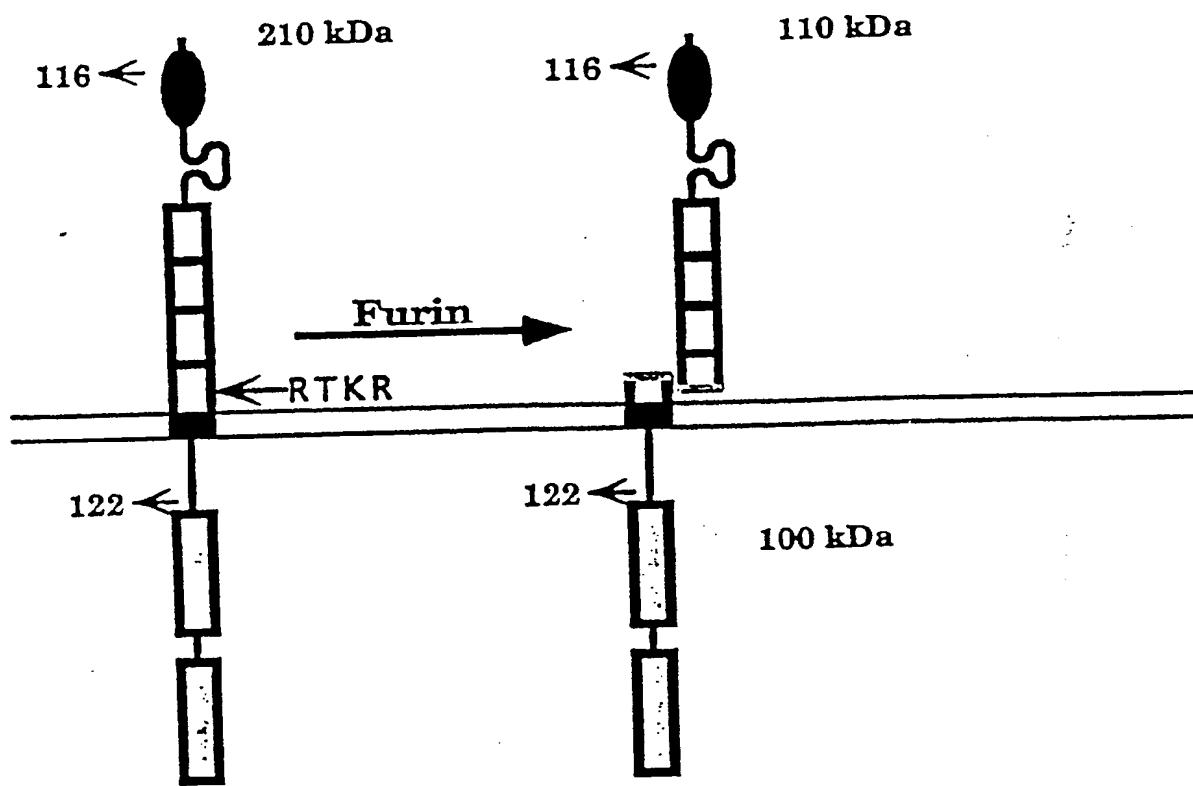


FIG. 14

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1 ATGGATAACGACTGCCGGCGGGCTGCCCTGCCTTCTGGCCCTCTCCTGGCCTCTCTGGATCGC 80
 1 M D T T A A A A L P A F V A L L L L S P W P L L G S A 27
 81 CCAAGGCCAGTTCTCCGAGGTGGCTGTACTTTGATGATGGTCCAGGGCCCTGTGATTACCAACAGGATCTGTATGATG 160
 27 Q G Q F S A G G C T F D D G P G A C D Y H Q D L Y D D 53
 161 ACTTTGAATGGGTCCATGTTACTGCTCAAGACCTCATTATCTACCAACCCACATGCCCAAGCTTCTATATGATAGTC 240
 54 F B W V H V S A Q E P H Y L P P E M P Q G S Y M I V 80
 241 GACTCTTCAGATCACGACCCCTGGAGAAAAAGCCAGACTTCAGCTGCCAACATGAAGGAGAACGACACTCACTGCATTGA 320
 81 D S S D H D P G E K A R L Q L P T M K E N D T H C I D 107
 321 TTTCAGTTACCTTATATAGCCAGAAAGGACTGAATCTGGCACTTGAAACATATTAGTTAGGGTQATAAAGGACCTC 400
 107 F S Y L L Y S Q X G L N P G T L N I L V R V N K G P L 133
 401 TTGCCAATCCAATTGGAATGTGACTGGATTCAACGGTAGAGATTGGCTTCGGGCTGAGCTAGCAGTGAACACCTTTC 480
 134 A N P I W N V T G P T G R D W L R A E L A V S T F W 160
 481 CCCAATGAATATCAGGTAATTTGAACTGAGCTGAAGCTCAGGGAGAGACTGGTTATATTGCCATTGATGACATCCA 560
 161 P N E Y Q V I F B A E V S G G R S G Y I A I D D I Q V 187
 561 ACTGAGTTATCCTTGTGATAAATCTCCTCATTTCTCCGTCTAGGGATGTAGGGTGAATGCAAGGGCAAAACGCTACAT 640
 187 L S Y P C D K S P B F L R L G D V E V N A G O N A T F 213
 641 TTCACTGCATTGCCACAGGGAGAGATGCTGCTGCAATAACAGTTATGGCTCCAGAGACGAATGGAGAACATACAGTA 720
 214 O C I A T C R D A V H N K L W L Q R R N G E D I P V 240
 721 GCCCAGACTAAGAACATCAATCATAGAACGTTGGCGCTTCCTCAGATTGCAAGAACGACTGACAAAAACTGACCAAGGATT 800
 241 A Q T K N I N H R R F A A S F R L Q E V T K T D Q D L 267
 801 GTATGGCTGTGAACTCAGTCAGAACGAGGTTCCGGTGTGCTCAACTTATTGTGAGAGAACCGCCAAAGAC 880
 267 Y R C V T O S E R G S G V S N F A Q L I V R E P P R P 293
 881 CCATTGCTCTCTCAGCTTCTGGTCTGGGCTACATATTGCTGATCCAACATAATGCCAACTCGATCATTGGCGAT 960
 294 I A P P Q L L G V G P T Y L L I Q L N A N S I I G D 320
 961 CCTCCTATCATCTGAAAGAACGAGTAGAGTACCGAATGACATCAGGATCTGGACAGAACCCATGCAGTCAATGCTCCAA 1040
 321 G P I I L K E V E Y R M T S G S W T E T H A V N A P T 347
 1041 TTACAAATTATGGCATTAGATCCAGATAACCGAATATGAGATCCAGTTCTACTTACAAGAACCTGGTGAAGGTGGAACGG 1120
 347 Y K L W H L D P D T E Y E I R V L L T R P G E G G T G 373
 1121 GGCTCCCAGGACCTCCACTAACCCAGAACAAATGTGCAGAACCTATGAGAACCCAAAGACATTAAAGATTGCTGAA 1200
 374 L P G F P L I T R T K C A E P M R T P K T L K I A E 400
 1201 ATACAGGAAAGACGGATTGCTGTGGACTGGAAATCTGGGTTACAAACATTACGGTTGCCAACACTTTATGTCACAT 1280
 401 I Q A R R I A V D W E S L G Y N I T R C H T F N V T I 427
 1281 CTGCTACCAATTACTTCCCTGGTCACAAACGAGAGCAAGGCAGACTTTGGACATGGACCCCCAAAGGCCCTCAGCATGTTG 1360
 427 C Y H Y F R G H N E S K A D C L D M D P K A P O H V V 453
 1361 TGAAACCATCTGCCACCTTATACAAATGTCAGCCTCAAGATGATCTTAACCAATCCAGAGGGAGGAACGGAGAGTGAAGAG 1440
 454 N H L P P Y T N V S L K M I L T N P E G R K E S E E 480
 1441 ACAATTATTCAAACTGATGAAGATGTGCCCTGGTCCCCTACAGTAATCTCTCAAGGAACATCCTTGAAGAACAT 1520
 481 T I I Q T D E D V F G P V P V K S L O G T S F E N K I 507
 1521 CTTCTTGAACTGGAAAGAACCTTGGATCCAATGGAATCATCACTCAATATGAGATCAGCTATGAGCTATAGATCAT 1600
 507 F L N W R E P L D F N G I I T Q Y E I S Y S S I R S F 533
 1601 TTGATCCTGCAGTCCCAGTGGCTGGACCTCCCCAGACTGTATCAAAATTATGGAACAGTACACACCAGTCTTATGCAT 1680
 534 D P A V P V A G F F C T V S N L W N S T H H V F M H 560
 1681 CTCCACCCCTGGAAACCACGTACCAAGTTCTCATAGAGCCAGCACGGTCAAAGGCTTTGGCCAGGCCACAGCCATCAATST 1760
 561 L H P G T T Y Q F F I E A S T V K G F G P A T A I N V 557
 1761 CACCAACAAATCTCAGCTCAACCTTACCTGACTATGAGGACTTGTGATGCCCTCTCTCAATGAACACTGCCACCCACAA 1840
 567 T T N I S A P T I I E Y E G V D A S L N E T A T T I T 611
 1841 CTGTATTGAGACCAAGGACCAACGAGCTCTCATCACTGCTTGTGAAACAGTGCACCCACAT 191
 614 V L L F F A C A Y S A I I C A Y C I V V E E L H I H 611

FIG 10 (1)

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1921	CGAACCAAGAGAGAAGCCGGAGCCATGGAATGCTACCAAGGTTCTGTCACATACCAAAATGCCATGACTGGGGTGCACC	2000
641	R T K R E A G A M E C Y O V P V T Y O N A M S G G A P	667
2001	GTATTACTTTGCTGCAGAACTACCCCCGGAAACCTACCTGAGCCTGCCCTTCACTGTGGGTGACAATCGGACCTACC	2080
667	Y Y F A A E L P P G N L P E P A P F T V G D N R T Y Q	693
2081	AAAGCCTTTGGAAACCCCTCCTTGGCTCCGGCAGAACATCTATTTCAGGGATGAGCAGTGTGGAGAAGGAA	2160
694	G F W N P P L A P R K G Y N I Y F Q A M S S V E K E	720
2161	ACTAAAAACCCACTGCGTACCCATTGCTACAAAGCAGAACAGAACAGAACAGAACAGAACAGAACAGAACAGAAC	2240
721	T K T O C V R I A T K A A T E E P E V I P D P A K O T	747
2241	AGACAGAGTGGTGAAGAAATAGCAGGAATTAGTGTGGAAATTGGTGTTCATCCTCCTCTCCACTTGTCAATTAAATTC	2320
747	D R V V K I A G I S A G I L V F I L L L L V V I L I V	773
2321	TAAGGAGGAAACTTGCTAAAGGAGGAAAGATGCCATGGGAATACCCGGCAGGAGATGACTCACATGGTGAATGCCA	2400
774	K K S K L A R K R K D A M G N T R Q E M T E M V N A	800
2401	ATGGATCGAAGTTATGCTGATCAGGCACTCTGCATGCAGAAAGATCCTTCCATCACCTCATGGACCACATAACTT	2480
801	H D R S Y A D Q S T L H A E D P L S I T P M D Q B N F	827
2481	TAGTCCAGATATGAGAACACAGTGTACAGCAGAGTCCAGTCCCTCTAGACGTACCTCGCTACCTCTGTGAGGGGA	2560
827	S P R Y E N H S A T A E S S R L L D V P R Y L C E G T	853
2561	CGGAATCCCTTACCAAGACAGGACAGCTCCATCCAGCCATCACGGTAGCTGATTACTGCAGCACATTAACTCATGAA	2640
854	E S P Y Q T G Q L H P A I R V A D L L Q H I N L M K	880
2641	ACATCAGACAGCTATGGTTCAGAGGAATATGAGAGCTTTGGAGGACAGTCAGCATCTGGGATGTAGCTAAAGGAA	2720
881	T S D S Y G F K E E Y E S P F E G Q S A S W D V A K K	907
2721	AGATCAAAATAGAGCAAAACCCGATATGAAACATTATAGCATATGATCACTCCAGAGTGTGATTTGCAACCGTAGAGG	2800
907	D Q N R A K N R Y G N I I A Y D H S R V I L Q P V E D	933
2801	ATGATCCTTCAGATTATATTAAATGCCAACTATATTGATGGTACCCAGAGAACACTCATTACATTGCAACCCAGGT	2880
934	D P S S D Y I N A N Y I D G Y Q R P S H Y I A T Q G	960
2881	CCCGTTCATGAAACAGTGTATGATTCTGGACCATTTGCCAAGAACATCTGCTTGCATTGTGATGGTACAAATT	2960
961	P V H E T V Y D F W R M I W Q E O S A C I V M V T N L	987
2961	AGTTGAGGTTGGCGGGTTAAATGCTATAAAATATTGGCCTGATGATACTGAAGTTATGGTGAATTCAAGTAACGTGTG	3040
987	V E V G R V K C Y K Y W P D D T E V Y G D F K V T C V	1013
3041	TAGAAATGCAACCACCTGCTGAATATGTTAGGACATTCAACCTGGAAAGGGGGGTACATGAAATCCGTGAAGTT	3120
1014	E M E P L A E Y V V R T F T L E R R G Y N E I R E V	1040
3121	AAACAGTTCCATTCACGGGCTGGCCCTGACCATGGAGTCCCTACCATGCTACAGGGCTGCTTCCATTCCGGCGACT	3200
1041	K Q F H F T G W P D H G V P Y H A T G L L S F I R R V	1067
3201	CAAGTTATCAAACCCCTCCAGTGTGGCCCATCTGTCACATTGCACTGGCTGGACAACTGGCTGCTACATTG	3280
1067	K L S N P P S A G P I V V H C S A G A G R T G C Y I V	1093
3281	TGATTGACATCATGCTAGACATGGCTGAAAGAGAGGGTGTGATATTACATTGTCACAGGCTTAAAGATCTCGG	3360
1094	I D I M L D M A E R E G V V D I Y N C V K A L R S R	1120
3361	CGTTAAATATGGTCCAGACAGAGGAACACTACATTATTCATGATGCCATTAGAACGCTGCTTATGTGGAGAAC	3440
1121	R I N M V Q T E E Q Y I F I H D A I L E A C L C G E T	1147
3441	TGCCATACCTGCTGTGAATTAAAGCTGCATATTGATGATGTTAGAACATAGACTCCAGACTAACTCTTCACATCTCA	3520
1147	A I F V C E F K A A Y F D M I R I D S Q T N S S H L K	1173
3521	AGGATGAATTTCAGACTCTGCAATTGCACTACACCCCTGCACTACAGCTGAAGACTGCACTATGGTGCCTGCCAAGGAAC	3600
1174	D E F O T L N S V T P R L O A E D C S I A C L P R N	1200
3601	CATGACAAGAACCGTTCTGGACATGCTGCCACCTGACAGATGTCTGCCATTAAATTACAATTGATGGGAGAGCAG	3680
1201	H D E K R F M D M L P F D R C L P F L I T I D G E S S	1227
3681	TAACATCAATGCTCTTATGGACAGCTACAGGCAACCCAGCTGCTTCTGTCACACAAATCCCTCTGCCAAACCA	3760
1227	N Y I N A A L M D S Y F C I A A F I V T C Y F L P N T	1253
3761	CTGTTAAAGCTTCTGGAGCATTAGTGTATGATTATGGCTGATGTTAAACGAAGTCGACTTGTCCAC	3840
1254	V K I F W R L V Y T Y G C T F I V M L N E V L L S C	1286

FIG. 15(2)

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3841	GGCTGCCCTCAGTACTGGCCAGAGGAAGGGATGCTACGATATGGCCCCATCCAACTGGAATGTATGTCTTGTCAATGGA	3920
1281	G C P Q Y W P E E G M L R Y G P I Q V E C M S C S M D	1307
3921	CTGTGATGTGATCAACCGGATTTAGGATATGCAATCTAACAAAGACCACAGGAAGGTTATCTGATGGTGCACAGTTTC	4000
1307	C D V I N R I F R I C N L T R P Q E G Y L M V Q Q F Q	1333
4001	AGTACCTAGGATGGCCTTCATCGAGAACTGCGCTGGATCCAAAAGGTCAATTCTGAAACTGATACTTCAGGTGGAAAAAC	4080
1334	Y L G W A S H R E V P G S K R S F L K L I L Q V E K	1360
4081	TGGCAGGAGGAATGCGAGGAAGGGGAAGGCCGGACGATTATCCACTGCCCTAAATGGTGGCGGGCAAGTGGCATGTTCTG	4160
1361	W Q E E C E E G E G R T I I H C L N G C C R S G M F C	1387
4161	TGCTATAGGCATGGTGTGAAATGGTCAACCGCAAAATGGTGTGATGTTTCCATGCAGTAAGACACTGAGGAACA	4240
1387	A I G I V V R M V K R O N V V D V F H A V K T L R N S	1413
4241	GCAAGCCAAACATGGTGGAAAGCCCCGGAGCAATACCGTTCTCCTATGATGTAGCTTGGAGTACCTGGAAATCATCTTAG	4320
1414	K P N M V E A P E Q Y R F C Y D V A L E Y L E S S	1439
4321	TTGGCTCACACTCTTAACTGCAATGAAAGAAACCTGTCATCTATTGAGCCAGCAGCTGTTGACCTGTTACACTT	4400
4401	GTCCAGAAAGATTTAAATGTCGGGGGGAGACTTTACATTGAGAGGTAAGTATTTATGAAAGTTGCTGTTAT	4480
4481	CTTAATAAAAAGAACTGAATTACTTTTATTACTATATTAAAGCATCAACATTTCATGCCACATTTAAATTATATTTAATA	4560
4561	AGAACCCAGATTGAAATGAGAACGTATTGGTGTACGTGAAACATGCCACCTTTCCATGGTTCAAGCTAGTGACCC	4640
4641	TACCAACATGTT	4651

(SEQ 10 NO: 4)

(SEQ 10
NO: 2)

FIG. 15(3)

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MCP7 HRPTP _u	MDTTAAALPAPVALLLSPWPLIGSAOGPSAGGCTPDDGPGACDTHODLYDDPEWVHSADEPHYLPPMPOGSMIV -MR LGTC - TL G -----TAGET - L EPYST G S SEG N EQ NTLTKPTSD W S L L	80 73
MCP7 HRPTP _u	DSSDRDPGEKARLOLPTMKENDTHC1DPSYLISORGPNPGTLN1LVRVNGPLANPIWN/TCP1GDRWLRAELAVSTPW NA CRPE OR R L OL B FVS KNSP L VY K N G IS DPT T N I	160 151
MCP7 HRPTP _u	PNEYQVIEAEGSGRSGYIAIDDIQVLSYPCDRSPHPLRLGDEVNAGONATFOCLATGRDAVHOKLWLRNNGEDIPV F V-ITS BO L EVK GH TIT ION P S I TWAGIR GIDVR A L	240 230
MCP7 HRPTP _u	ACTIONINERBPAISPRLOEVTKTDDOLYRCVTOSERGSGVSNPAOLIVREPPRPLAPPOLLGVGPTYLLIGLNANSICGD KEY VESS I NWNT R ACK MIRT G V I YEVK V AS A W N	320 310
MCP7 HRPTP _u	GPIILKEVEYRMTSGSWTETHAVNAPTYKMLDPTDEYEIRVLLTRPGEOGTGLPPPLITRTRCAEPHRTPTKTRIAE VAR CTA NEROP DSTS IG S S AR D G R K EVV	400 390
MCP7 HRPTP _u	YOAARIAVDNEISLGYNITRCHTFNVTICYBFRGNE--SKACLDMDPRAPOHVNVHLPYTNVSLRMLINPREGRES VKS O TIR PP V SY L VE C OV OO OVREEVSW TENS H TITN S V L M	478 470
MCP7 HRPTP _u	EETI IOTDEDVPGPVPVKSLOOTSEKNIPLNWRKELDPLNGLIOTYELSYSSISRSPDPAVPVGPPOTVSNLMNSTHGP O L V L A T E I S T E O R T O T Y V L T R A V S EIDLSNOSGR K C E F L	558 550
MCP7 HRPTP _u	MRLHPOTTYOPPIRASTVKGP0PATAINVTTINISAPTLPDTEYEGVADASNETATTITVLLRPAQAKAPISAYGIVVEELH PG Y S T A PATNOF K SM A -LETP Q DN V M K MSR V V ER	638 629
MCP7 HRPTP _u	PERTRKREAGAMECYOVPVYTORANSOGAPTYPAALPPGNLPEPAPPYVGDNRTYOGPNNPLAPRRCGYNITYOAMSSVE R KTEILK P IHP SLMBO P ADS QAO I K N Y T L Y S R A RAN	718 709
MCP7 HRPTP _u	KETKTCVRIATKAATEEPEVIPDAPROTIVVLAGISAGILVPIILLVVIKKSILAKRUDANGNTROENTH G ID OV G A-T KFV E E BT VI L VII F G V VM R ET SS V	798 788
MCP7 HRPTP _u	NAMDRSYADOSTLHAEDPLSITPMDOHNPSPRY-----ENHSATAESSRLLOVPRY-LCE S K E G -NCDEAP -- T LNG SVSSPSSPTMKTNTLSTSVPNSTYD T THASD T S VOSHT KUR	852 865
MCP7 HRPTP _u	GTESPYOTGOLIPPAIRVADLLOHDLNLMKTSDSYCPKEEYESPPECOSASWDJKDONRAKNTYQNLAYDERSVILCPV PADV TO CAEG P S E M R TI	932 945
MCP7 HRPTP _u	EDDPSSDYINANYIDGYQRPSEHYIATOGPVRHETVYDWRMHWQE0SACIVMVTNUVEVGRVKCYKWPDDTEVYGDPRKVT G TN G B N M O I V B N T S I C I K I	1012 1025
MCP7 HRPTP _u	CVEMEFLAETVVRPTPLERGYNIEIREVKOPHTGMDHGVPYHATGILSPIRRVLKLSNPPSAGPIVVCAGAGRTOCY LI T L I AV K VB IR C V Q S K E L P	1092 1105
MCP7 HRPTP _u	IVIDIMLDMAERGVVDIYNCVKALRSRRINMVOTEEQYIPIHDAILEACLCGETAIPVCEPRAAYPDHMRIDSOTNSH RE V V D S V ASQVSL Y NKL P O	1172 1185
MCP7 HRPTP _u	LKDEPOTLNSVTPLAOEDCSIACLPRNHDIONRPMNLPPDRCLPPLITIDGESSNYINAALMDSYROPAAPIVTOYPLP I E R M T RV L E C I R S H	1252 1265
MCP7 HRPTP _u	NTVKDPWRLVYDYGCTSIVMLNEVDSOOGCPOWPEEGMLRYGP1OVECMSCSMDCDVINRIPRIONLTRPCEGYLMVQO L B V D PA L N V B H FV ADLEE I S Y AA D R	1332 1345
MCP7 HRPTP _u	POTLGWASEREVPGSKRSPLLELIOVEKWOEECEDEGRTIIECLNGGRSGMPCAIGTVVEMVKRONVVDVPHAVKTLR P PHY DT V R D YNG P VV T S C L R H RT	1412 1425
MCP7 HRPTP _u	NSKPNMVEAPEOYRFCYDVALEYLESS* N DLLD R E N G*	1435 1452

FIG. 16

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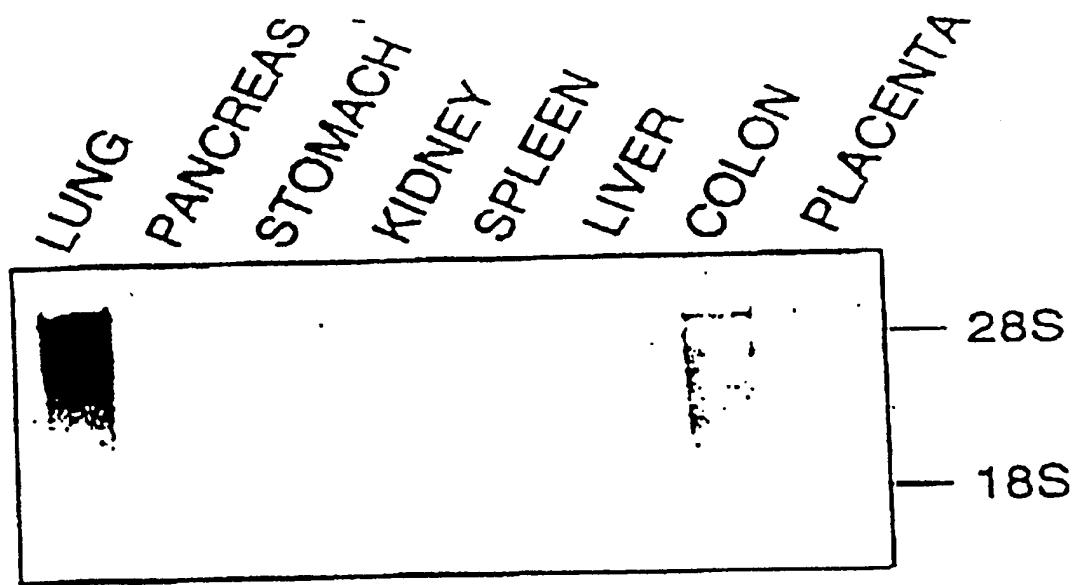


FIG. 17

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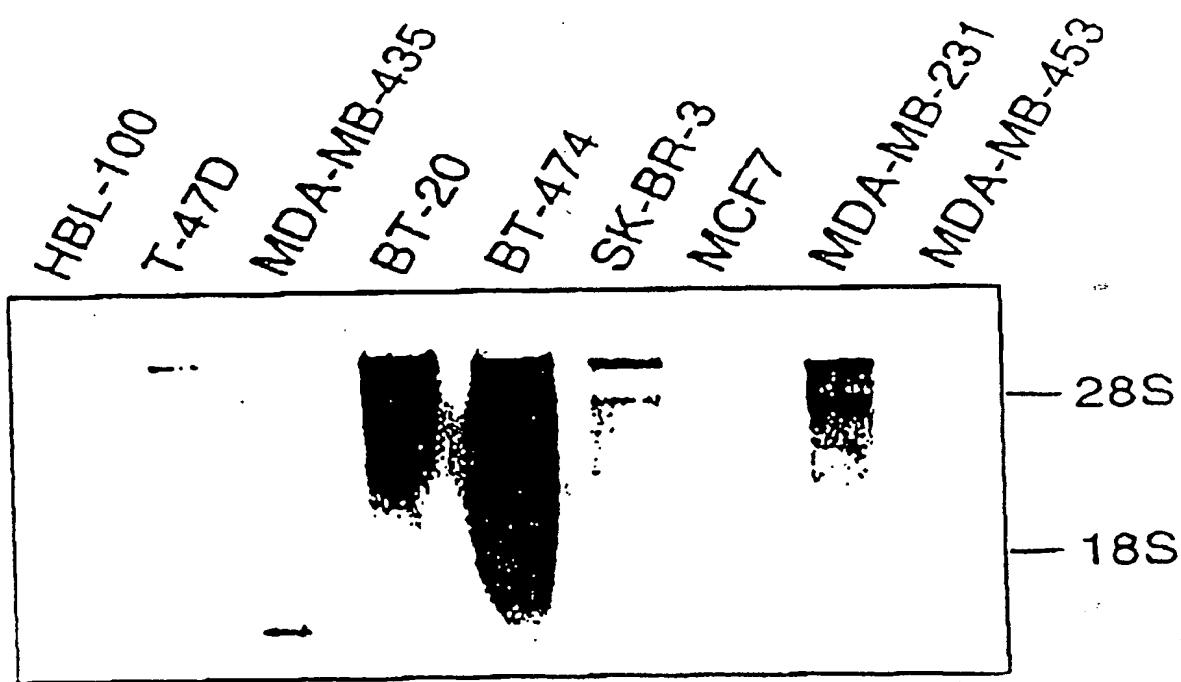


FIG. 18

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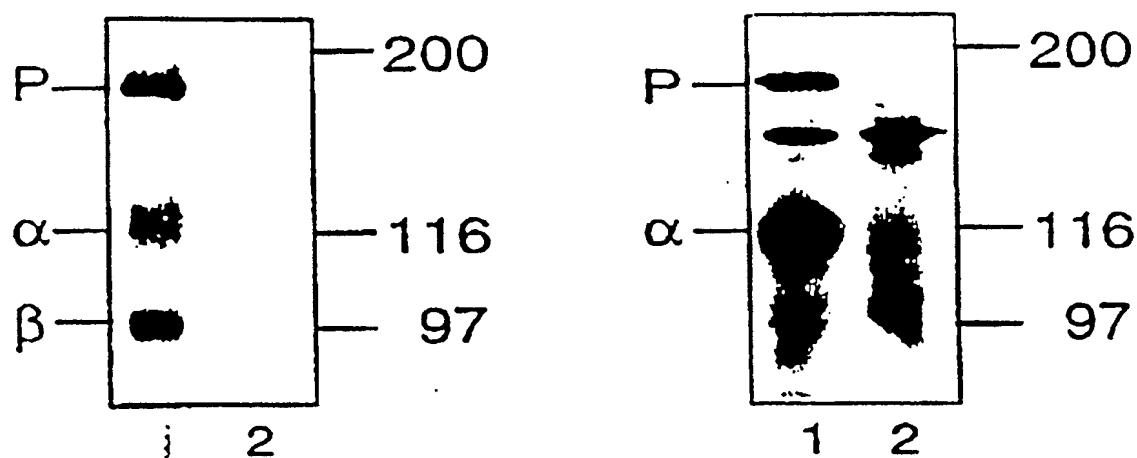


FIG. 19

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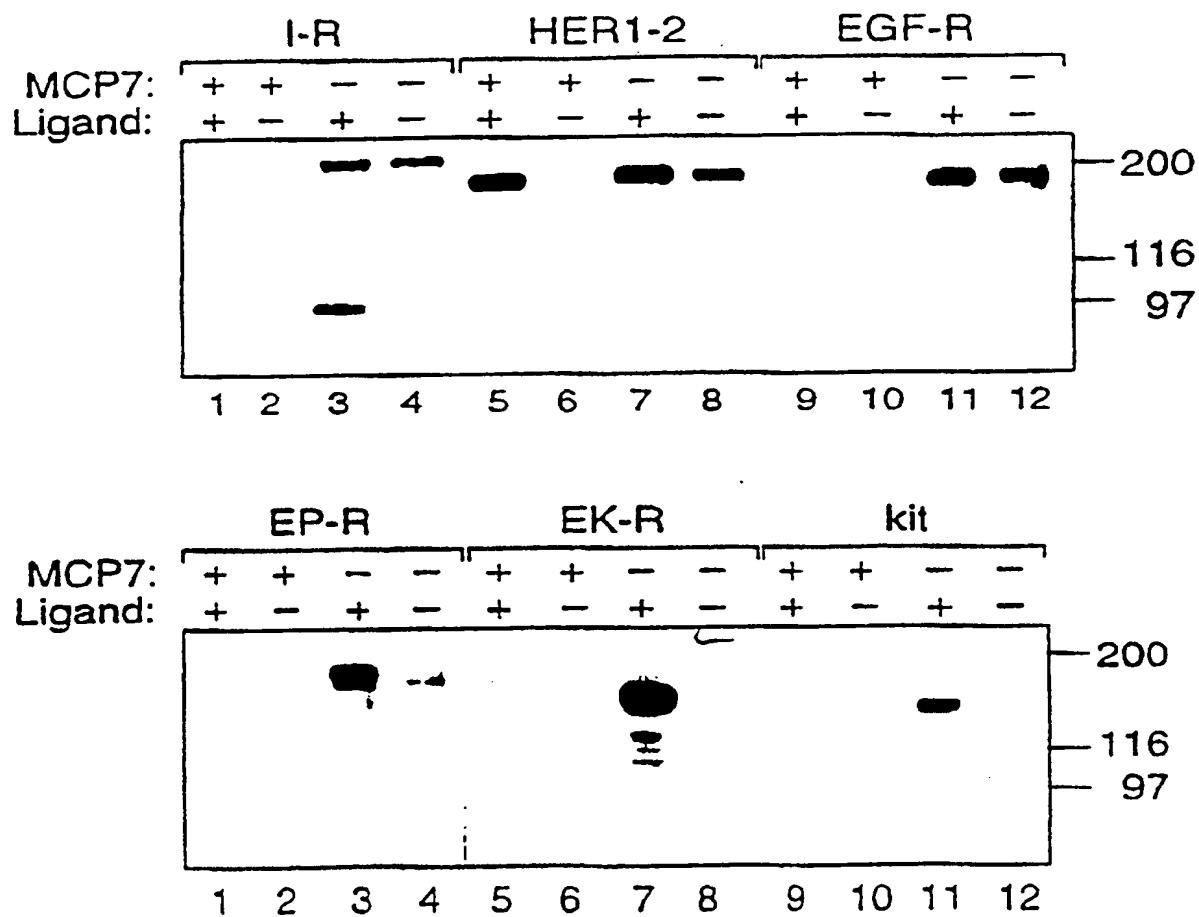


FIG. 20

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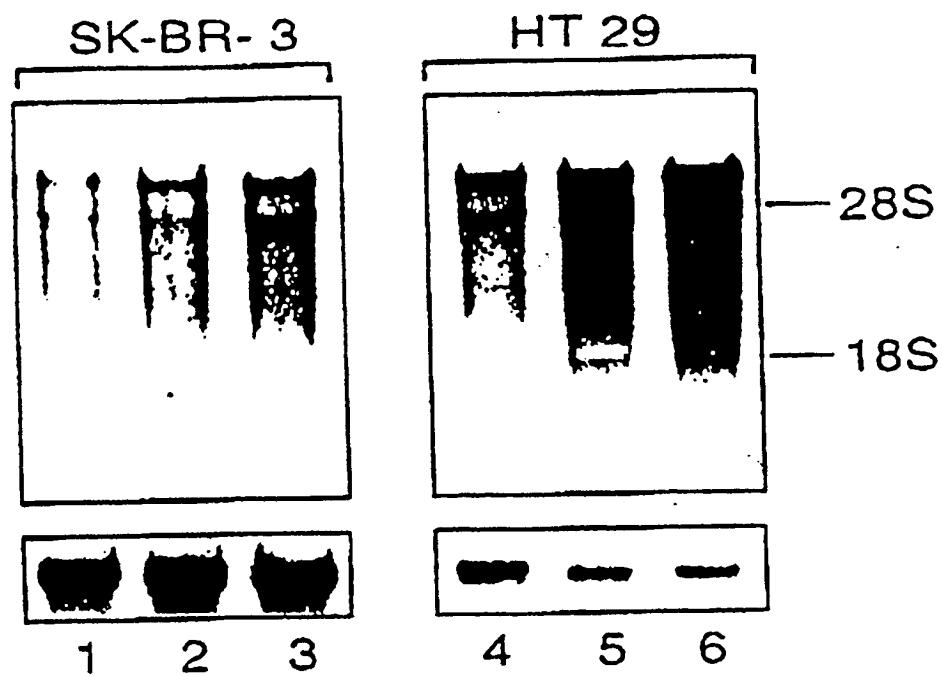


FIG. 21

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1 2 3 4 5

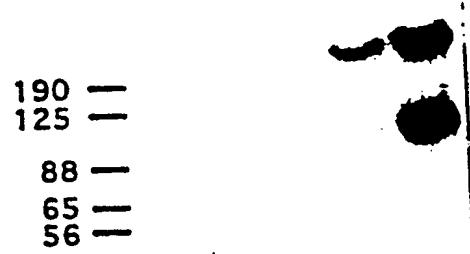
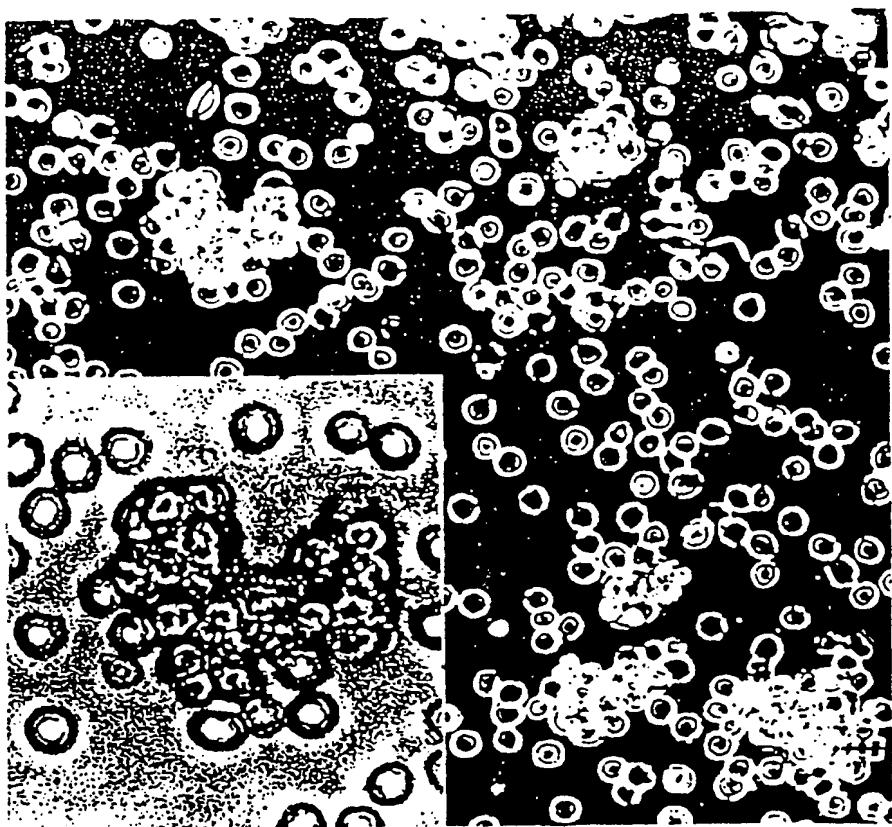


FIG. 22 A

R-PTP- κ 

Control

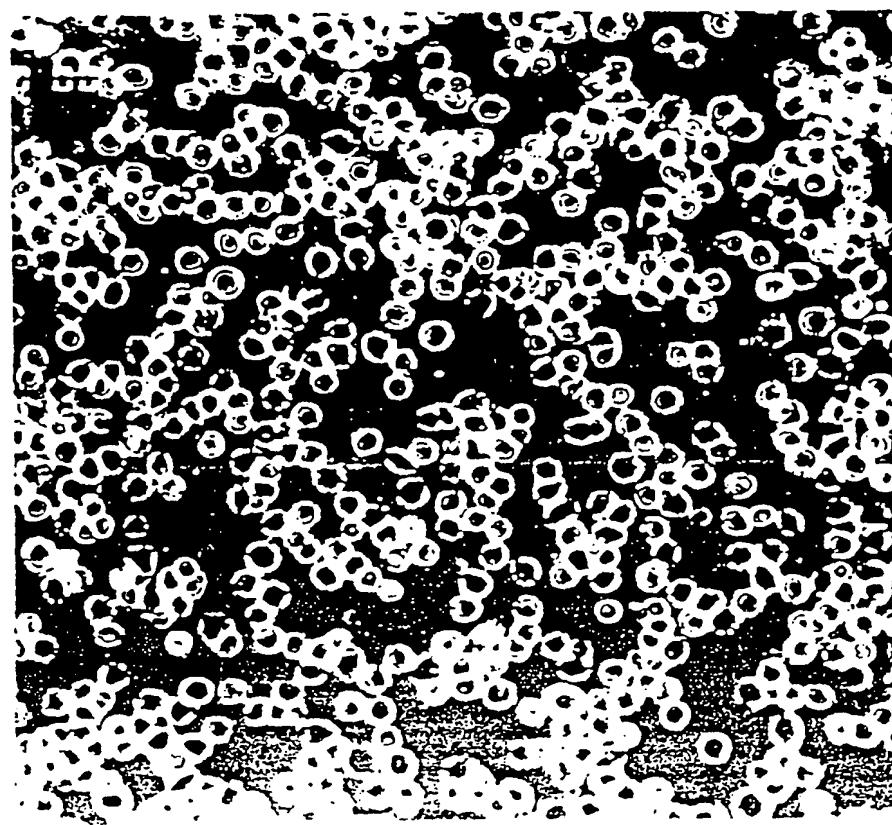


FIG. 22 B

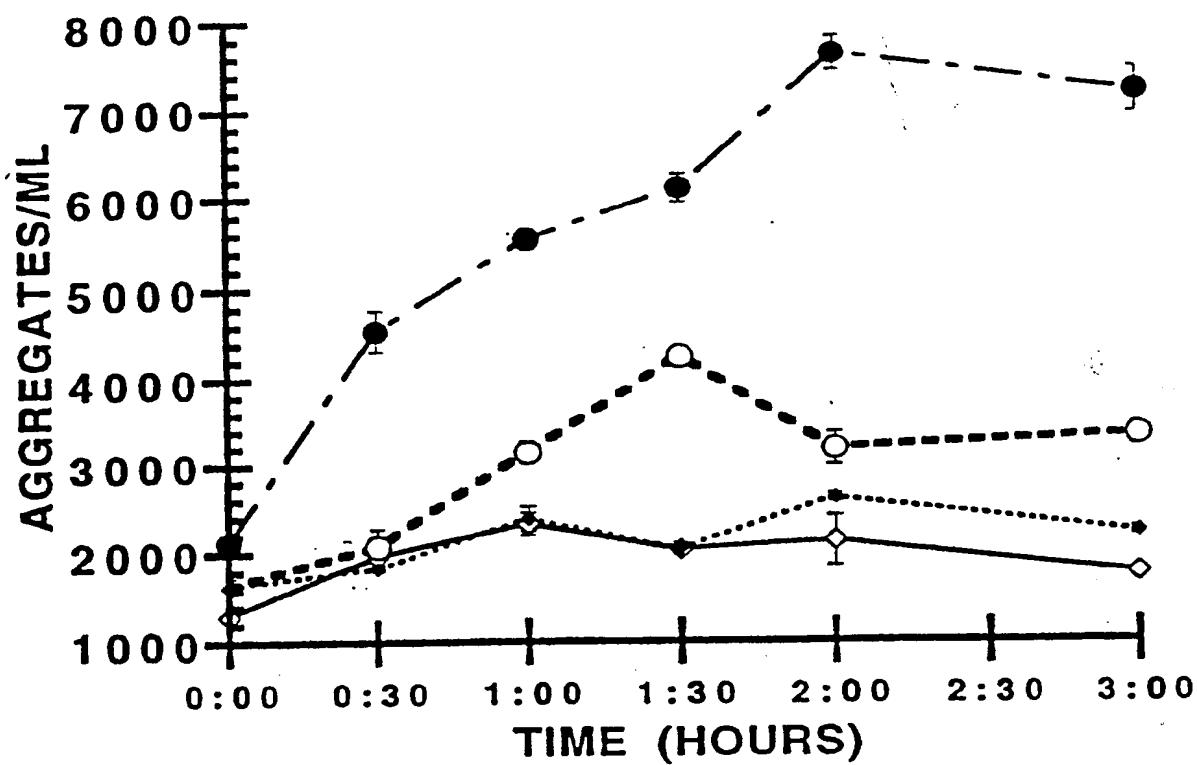


FIG. 22 C

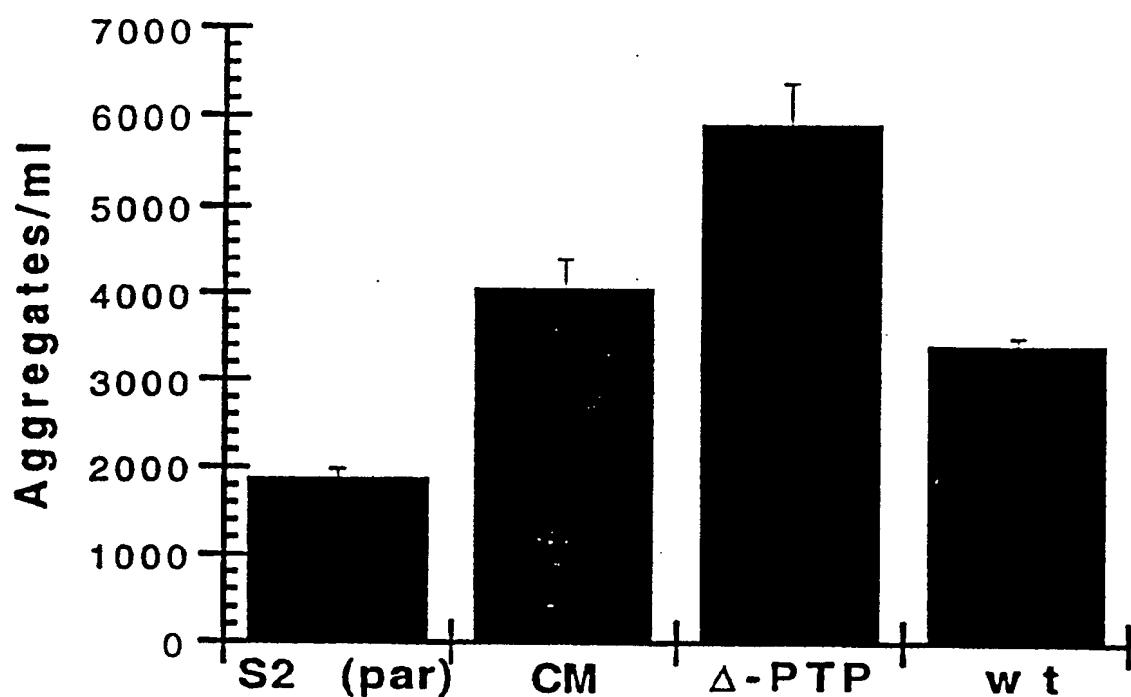


FIG. 22 D

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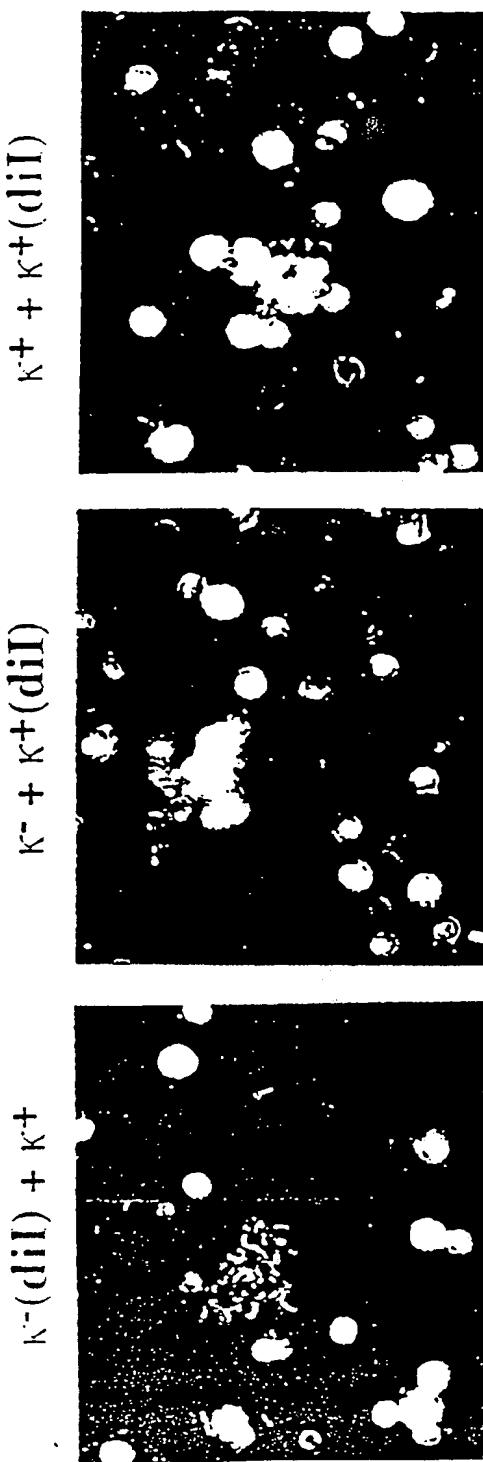


FIG. 23

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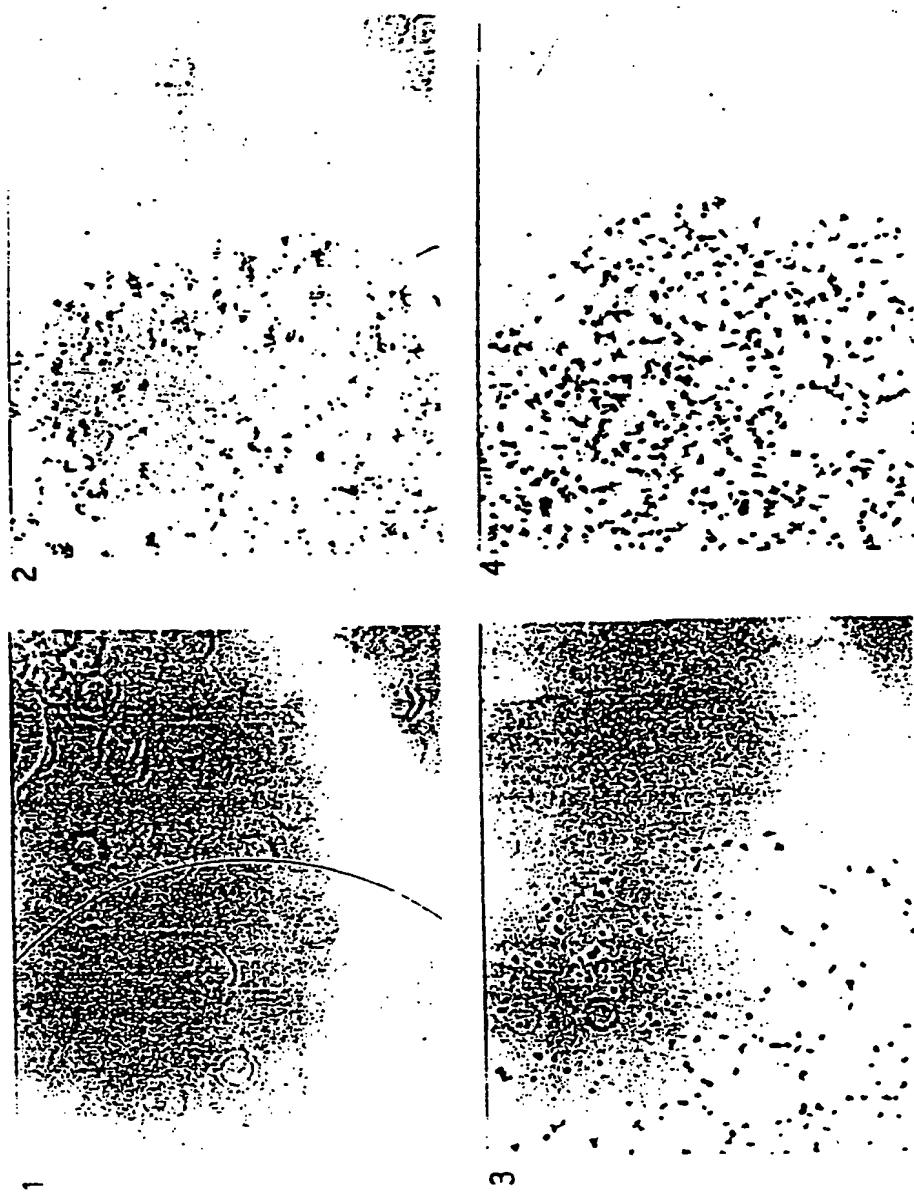


FIG. 24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04377

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C07K 13/00; C12N 15/00; C12P 21/02; A61K 37/02
US CL :536/23.1; 530/350, 388.1; 435/ 69.1, 320.1; 514/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 530/350, 388.1; 435/ 69.1, 320.1; 514/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, MEDLINE, WPI
search terms: receptor protein tyrosine phosphatase, RPTP-kappa

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P ----- Y, P	Molecular and Cellular Biology, Volume 13, Number 5, issued May 1993, Jiang et al, "Cloning and Characterization of R-PTP κ , a New Member of the Receptor Protein Tyrosine Phosphatase Family with a Proteolytically Cleaved Cellular Adhesion Molecule-Like Extracellular Region", pages 2942-2951, see pages 2943-2946.	1-19 ----- 20-48
Y	The EMBO Journal, Volume 9, Number 10, issued 1990, Krueger et al, "Structural Diversity and Evolution of Human Receptor-Like Protein Tyrosine Phosphatases", pages 3241-3252, see pages 3242-3245.	1-48

Further documents are listed in the continuation of Box C.



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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*&*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

02 AUGUST 1994

Date of mailing of the international search report

16 AUG 1994

Name and mailing address of the ISA/US
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Box PCT
Washington, D.C. 20231

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Authorized officer

Sally Teng

Telephone No. (703) 308-0196

S. Teng for

INTERNATIONAL SEARCH REPORT

National application No.
PCT/US94/04377

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Federation of European Biological Societies, Volume 290, Number 1,2, issued September 1991, Gebbink et al, "Cloning, Expression and Chromosomal Localization of a New Putative Receptor-Like Protein Tyrosine Phosphatase", pages 123-130, see pages 124-125.	1-22
Y	Science, Volume 253, issued 26 July 1991, Fischer et al, "Protein Tyrosine Phosphatases: A Diverse Family of Intracellular and Transmembrane Enzymes", pages 401-406, see pages 402-405.	1-48
Y	Proceedings of National Academy of Science, USA, Volume 80, issued September 1983, Hoffman et al, "Kinetics of Homophilic Binding by Embryonic and Adult Forms of the Neural Cell Adhesion Molecule", pages 5762-5766, see page 5762.	28-45

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